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Creatine transporter deficiency: molecular and functional tools for diagnosis, and prevalence of this X-linked mental retardation syndrome

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Creatine transporter deficiency: molecular and functional tools for diagnosis, and prevalence of this X-linked mental retardation syndrome

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ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. L.M. Bouter,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der Geneeskunde
op maandag 20 november 2006 om 13.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

Efraim Hananya Rosenberg

geboren te Jeruzalem, Israël

promotor: prof.dr.ir. C.A.J.M. Jakobs
copromotor: dr. G.S. Salomons

“Om vrede te hebben met dit rare leven;
om te aanvaarden wat wij niet begrijpen;
om rustig af te wachten wat ons te wachten staat;
moet je wijzer zijn dan ik ben.”

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Voor Ophira, Elitsoer, Reëlaja & Naäma,
Voor mijn ouders.

Abbreviations

ADP	adenosine diphosphate
AGAT	L-arginine:glycine amidinotransferase
ARX	Aristaless related homeobox gene
ATP	adenosine triphosphate
CCDS	cerebral creatine deficiency syndrome
CDS	coding sequence
CI	confidence interval
CK	creatine kinase
Cr	creatine
Cr:Crn	creatine/creatinine ratio
Crn	creatinine
DDSN	Department of Disabilities and Special Needs
EGFP	enhanced green fluorescence protein
FMR1	fragile-X mental retardation gene 1
fraX	fragile-X syndrome
GAMT	guanidinoacetate methyltransferase
GCMS	gas chromatography mass spectrometry
H-MRS	proton magnetic resonance spectroscopy
HPLC	high-performance liquid chromatography
IVS	intervening sequence
MIM	Mendelian inheritance in man
MR	mental retardation
MRS	magnetic resonance spectroscopy
ORF	open reading frame
SD	standard deviation
SDM	site directed mutagenesis
SID-GCMS	stable isotope dilution gas chromatography mass spectrometry
SLC6A8	solute carrier family 6, member 8
TM	transmembrane domain
XLMR	X-linked mental retardation

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Chapter 1

Outline of the thesis.

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Outline of the thesis

Creatine and phosphocreatine play essential roles in the storage and transmission of phosphate-bound energy [1,2]. Creatine kinase catalyses the phosphorylation and dephosphorylation of creatine and phosphocreatine, respectively, providing a high energy phosphate buffering system [3]. Creatine and phosphocreatine are small, and less negatively charged molecules than ATP and ADP, and can be stored intracellular at a much higher concentration, allowing for a high intracellular flux of high-energy phosphates.

In the human body, the creatine pool is maintained by creatine biosynthesis (50%) and nutritional uptake (50%). Creatine biosynthesis involves a two-step reaction: the first is catalysed by L-arginine:glycine amidinotransferase (AGAT; EC 2.1.4.1) and results in the formation of guanidinoacetic acid (GAA), which is subsequently methylated by guanidinoacetate methyltransferase (GAMT; EC 2.1.1.2) resulting in the formation of α-methylguanidino acetic acid, named creatine. Intracellular, creatine and phosphocreatine are converted non-enzymatically to creatinine with a constant daily turnover of 1.5% of the creatine pool.

Cellular transport of creatine is of fundamental importance for creatine homeostasis in tissues void of creatine biosynthesis. Creatine is distributed via the blood stream and taken up via the creatine transporter. The creatine transporter gene (*SLC6A8/CT1/CRTR1*) has been mapped to Xq28 [4]. The *SLC6A8* gene spans ~8.4 kb and consists of 13 exons (GenBank accession no. Z66539) and encodes a protein of 635 amino acids with a predicted molecular weight of 70kDa [5]. SLC6A8 is a member of the Na^+/Cl^--dependent neurotransmitter transporter family, being most closely related to the GABA/taurine/betaine transporter subfamily (46-53% aminoacid sequence identity). The genes of the neurotransmitter transporter gene family encode glycoproteins with 12 putative transmembrane regions that mediate Na^+ dependent re-accumulation of released neurotransmitters into presynaptic terminals [6] (Figure 1).

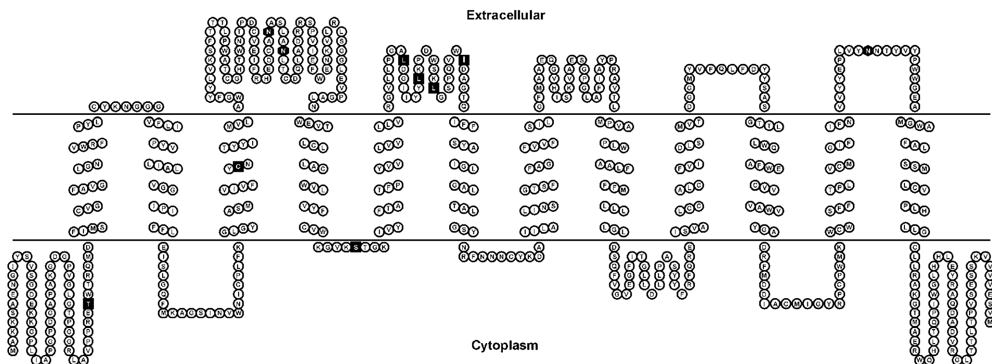

Figure 1. Putative 12 transmembrane structure of SLC6A8. Putative N-glycosylation sites, cAMP-PK phosphorylation sites, Leu-zipper and Cys-144 (close to a substrate-binding site) are depicted as highlighted amino acids [7,8].

In 2001, a six-year-old boy was examined who presented with a marked reduction of the creatine signal in the brain on proton magnetic resonance spectroscopy (MRS). Conversely, the urinary creatine/creatinine ratio was high. Clinical findings were mental retardation, autistic like behavior, speech and language delay and partial status epilepticus. The family history of mental retardation in males and behavioral problems in females, was suggestive for X-chromosomal inheritance of the trait [9]. The unbalanced compartmentalization of creatine in the body, the putative X-linked inheritance and the fact that the gene for a creatine transporter was mapped at the X-chromosome - Xq28, lead to the identification of a pathogenic nonsense mutation in the creatine transporter gene - *SLC6A8* [10]. The creatine uptake in fibroblasts of the patient was shown to be impaired as compared to that in control fibroblasts. Thus, a novel cerebral creatine deficiency syndrome was discovered at the Metabolic Unit of the Dept. of Clinical Chemistry of the VUmc in Amsterdam in collaboration with the Cincinnati Children's Hospital Medical Center in the USA. [9,10]. In a relatively short time span, more unrelated families were identified with SLC6A8 deficiency, including several within one metropolitan area. The common hallmarks are mental retardation, speech and language delay, an elevated urinary creatine/creatinine ratio, and a mutation in *SLC6A8* [10].

The diagnosis of SLC6A8-deficiency in unrelated families shortly after the recognition of this defect, raised the following questions.

- Is impaired creatine uptake in deficient primary fibroblasts complemented by introduction of the *SLC6A8* wild-type coding sequence?
- What is the prevalence of SLC6A8-deficiency?
- Can we characterize unclassified missense variants in the *SLC6A8* gene found in mentally retarded patients?
- Can better diagnostic recommendations be established for the detection of SLC6A8-deficiency and other cerebral creatine deficiency syndromes?

The high urinary creatine/creatinine ratio, the marked reduction of the cerebral creatine signal on proton MRS, the X-linked inheritance pattern and mutations in the *SLC6A8* gene strongly suggested that SLC6A8-deficiency is primarily caused by mutations in the *SLC6A8* gene. However, to provide final proof, the SLC6A8 coding region was stably transfected into SLC6A8-deficient fibroblasts, resulting in the complete restoration of creatine uptake in these cells (**chapter 2**).

Next, we thought to investigate the prevalence of SLC6A8-deficiency. Based on the main clinical hallmark, X-linked mental retardation, DNA samples of 288 males with X-linked mental retardation from the European XLMR consortium were screened for the presence of *SLC6A8* mutations (**chapter 3**).

Since the group of X-linked mentally retarded patients is estimated to represent only 10% of all mentally retarded patients, we conducted a subsequent prevalence study in an unselected panel of mentally retarded patients, in collaboration with the Greenwood Genetic Center (USA). In this study, DNA samples of 488 males with idiopathic mental retardation were analyzed for *SLC6A8* mutations (**Chapter 4**).

The whole spectrum of *SLC6A8* mutations, found in SLC6A8-deficient patients comprises complete and partial gene deletions, single amino acid deletions, splice errors, nonsense mutations and missense mutations. The latter type of mutations is particularly difficult to classify. Therefore, we developed overexpression assays to investigate the nature of such variants (**chapter 5**).

The characteristics and the clinical phenotype of patients, identified in the screening of the cohort of chapter 3, as well as patients described in the literature are summarized in **chapter 6**.

SLC6A8 deficiency belongs to a group of cerebral creatine deficiency syndromes (CCDS). In **chapter 7** an overview on the knowledge of SLC6A8 deficiency as well as the other CCDS, AGAT en GAMT deficiencies, is provided including diagnosis, clinical characteristics and the recently identified *in vitro* data on creatine function in the brain.

The studies described in this thesis are summarized and discussed in **chapter 8**, including the implications for their diagnosis. The chapter concludes with a glance on future developments in CCDS.

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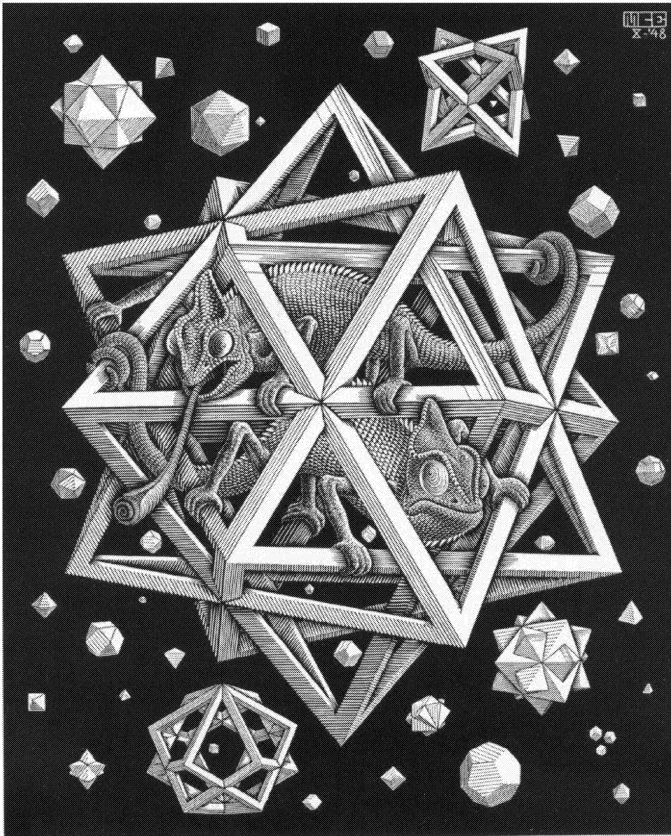
Chapter 2

Overexpression of wild-type creatine transporter (SLC6A8) restores creatine uptake in primary SLC6A8-deficient fibroblasts.

Journal of Inherited Metabolic Disease (2006) ***29****(2-3), 345-346*



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DOI 10.1007/s10545-006-0271-6

SSIEM SYMPOSIUM 2005

Overexpression of wild-type creatine transporter (SLC6A8) restores creatine uptake in primary SLC6A8-deficient fibroblasts

Efraim H. Rosenberg · Cristina Martínez Muñoz · Ton J. Degrauw · Cornelis Jakobs · Gajja S. Salomons

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Summary In the study reported, we prove that mutations in the *SLC6A8* gene are responsible for SLC6A8 deficiency, a cerebral creatine deficiency syndrome (CCDS), since overexpression of the wild-type SLC6A8 open reading frame (ORF) restores the creatine uptake profile in SLC6A8-deficient fibroblasts.

Background

SLC6A8 deficiency, a cerebral creatine deficiency syndrome (CCDS), is caused by mutations in the creatine transporter gene (*SLC6A8*; McKusick 300036) (Nash et al 1994; Rosenberg et al 2004; Salomons et al 2001). The affected males present with X-linked mental retardation (XLMR), expressive speech and language delay, epilepsy, developmental delay and autistic behaviour (deGrauw et al 2002; Kleefstra et al 2005; Mancini et al 2005; Salomons et al 2003). Affected males have a reduction of the creatine signal in proton magnetic resonance spectroscopy (^{1}H MRS) of the brain, increased urinary creatine/creatinine ratio and have impaired creatine uptake in cultured primary fibroblasts. According to skewed X-inactivation, females with a heterozygous mutation may be completely asymptomatic whereas others may exhibit pronounced biochemical and clinical manifestations similar to the male phenotype. In order to provide final proof that SLC6A8 deficiency is caused by a defect of the creatine transporter, we transfected the wild-type SLC6A8 open reading frame (ORF) into primary fibroblasts of a SLC6A8-deficient patient, and tested the creatine uptake capacity.

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Competing interests: None declared

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Methods

Wild-type SLC6A8 ORF was cloned into a pEGFP-N1 expression vector in frame with the enhanced green fluoresent protein (EGFP) sequence. This construct was then stably transfected into primary fibroblasts of a SLC6A8-deficient patient. These fibroblasts are deficient in creatine uptake and contain a nonsense mutation (c.1540C>T; p.Arg514X) as described previously (Salomons et al 2001). Expression of the SLC6A8-EGFP fusion protein or the EGFP (empty vector) protein was confirmed in the stable transfectants by the presence of green fluorescence signal as detected by FACS analysis. Furthermore, expression of the SLC6A8-EGFP mRNA was shown by RT-PCR. Subsequently, transfected cells were incubated for 24 h with 25 μmol/L creatine, 500 μmol/L creatine or 500 μmol/L creatine and 500 μmol/L guanidinopropionate (creatine transporter inhibitor). The cells were harvested by trypsinization and stored as dry pellets at −80 °C until further use. Intracellular creatine content was measured by stable-isotope dilution GC-MS.

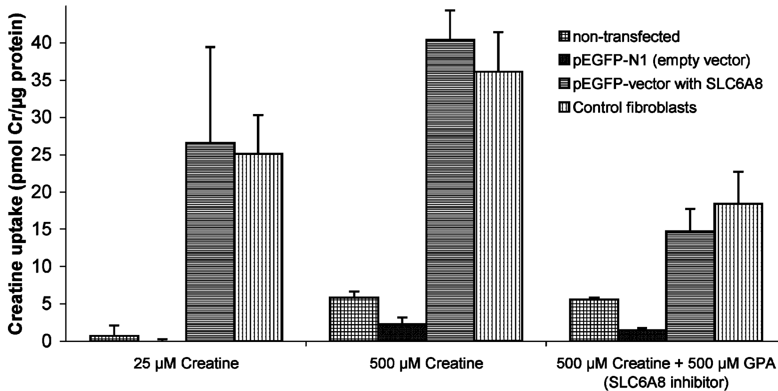

Fig. 1 Overexpression of wild-type SLC6A8-EGFP restores creatine uptake in primary SLC6A8/creatine transporter-deficient fibroblasts (c.1540C>T; p.Arg514X; Salomons et al 2001). Stably transfected cells and control fibroblasts were incubated in triplicate for 24 h with 25 µmol/L creatine, 500 µmol/L creatine or 500 µmol/L creatine and 500 µmol/L guanidinopropionate (creatine transporter inhibitor). Intracellular creatine content was measured by stable-isotope dilution GC-MS. Error bars represent the standard error of mean of triplicate measurements

Results

Transfection of deficient primary fibroblasts with wild-type SLC6A8 ORF results in restoration of the creatine uptake profile to comparable with that of control fibroblasts, in contrast to mock transfectants (see Fig. 1). In order to study the nature of variants that have been detected in patients, these variants are currently being introduced into the SLC6A8-EGFP construct, transfected into deficient primary fibroblasts, and analysed as described.

Conclusion

We provide definitive proof that mutations in the *SLC6A8* gene are responsible for SLC6A8 deficiency, a CCDS, since overexpression of the wild-type SLC6A8 ORF restores the creatine uptake profile in SLC6A8-deficient fibroblasts. This approach will be used to classify the effects of novel *SLC6A8* sequence variants of unknown significance (i.e. missense mutations).

Acknowledgement The research was supported by grant VIDI 917.56.349 from the Dutch Organization for Scientific Research (NWO/ZonMW).

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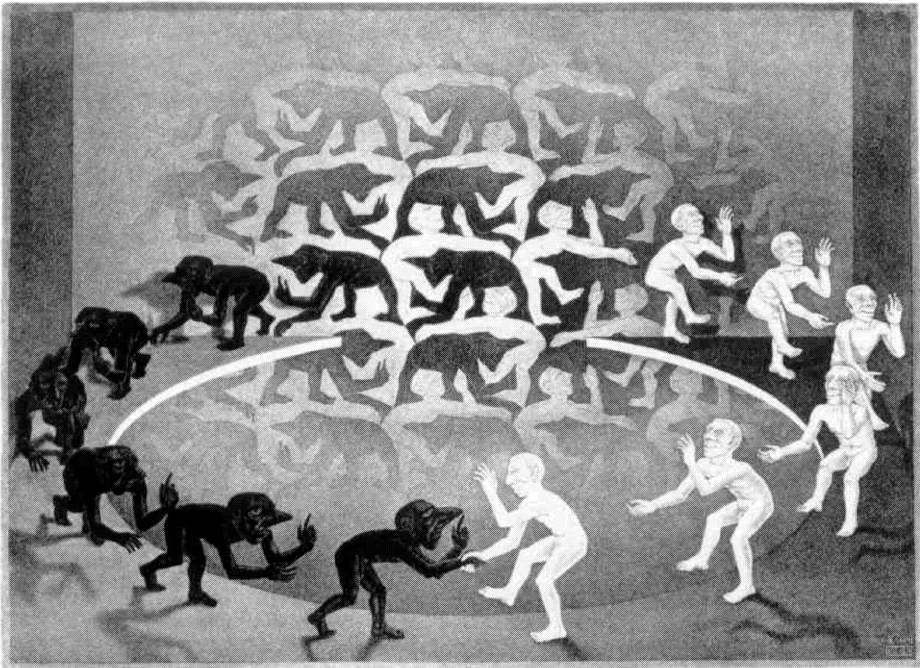
Salomons GS, van Dooren SJ, Verhoeven NM, et al (2001) X-linked creatine-transporter gene (SLC6A8) defect: a new creatine-deficiency syndrome. *Am J Hum Genet* **68**(6): 1497–1500.

Salomons GS, van Dooren SJ, Verhoeven NM, et al (2003) X-linked creatine transporter defect: an overview. *J Inherit Metab Dis* **26**(2–3): 309–318.

Chapter 3

High Prevalence of SLC6A8 Deficiency in X-Linked Mental Retardation.

*American Journal of Human Genetics (2004) **75**(1), 97-105*



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Am. J. Hum. Genet. 75:97–105, 2004

Report

High Prevalence of SLC6A8 Deficiency in X-Linked Mental Retardation

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A novel X-linked mental retardation (XLMR) syndrome was recently identified, resulting from creatine deficiency in the brain caused by mutations in the creatine transporter gene, *SLC6A8*. We have studied the prevalence of *SLC6A8* mutations in a panel of 290 patients with nonsyndromic XLMR archived by the European XLMR Consortium. The full-length open reading frame and splice sites of the *SLC6A8* gene were investigated by DNA sequence analysis. Six pathogenic mutations, of which five were novel, were identified in a total of 288 patients with XLMR, showing a prevalence of at least 2.1% (6/288). The novel pathogenic mutations are a nonsense mutation (p.Y317X) and four missense mutations. Three missense mutations (p.G87R, p.P390L, and p.P554L) were concluded to be pathogenic on the basis of conservation, segregation, chemical properties of the residues involved, as well as the absence of these and any other missense mutation in 276 controls. For the p.C337W mutation, additional material was available to biochemically prove (i.e., by increased urinary creatine:creatinine ratio) pathogenicity. In addition, we found nine novel polymorphisms (IVS1+26G→A, IVS7+37G→A, IVS7+87A→G, IVS7-35G→A, IVS12-3C→T, IVS2+88G→C, IVS9-36G→A, IVS12-82G→C, and p.Y498) that were present in the XLMR panel and/or in the control panel. Two missense variants (p.V629I and p.M560V) that were not highly conserved and were not associated with increased creatine:creatinine ratio, one translational silent variant (p.L472), and 10 intervening sequence variants or untranslated region variants (IVS6+9C→T, IVS7-151_152delGA, IVS7-99C→A, IVS8-35G→A, IVS8+28C→T, IVS10-18C→T, IVS11+21G→A, IVS12+15C→T, *207G→C, IVS12+32C→A) were found only in the XLMR panel but should be considered as unclassified variants or as a polymorphism (p.M560V). Our data indicate that the frequency of *SLC6A8* mutations in the XLMR population is close to that of CGG expansions in *FMR1*, the gene responsible for fragile-X syndrome.

Creatine and phosphocreatine play essential roles in the storage and transmission of phosphate-bound energy (Walker 1979; Wyss and Kaddurah-Daouk 2000). Humans maintain their creatine pool by creatine biosynthesis, which involves two enzymes—L-arginine:glycine amidinotransferase (AGAT; Enzyme Commission [EC] number 2.1.4.1) and guanidinoacetate methyltransferase (GAMT; EC 2.1.1.2)—as well as nutritional uptake. Cellular transport is of fundamental importance for creatine homeostasis in tissues void of creatine biosynthesis. The creatine transporter gene (*SLC6A8/CT1/CRTR1* [MIM 300036]) has been mapped to Xq28 (Gregor et al. 1995) and is a member of the solute-carrier family 6 (neurotransmitter transporters). The *SLC6A8* gene spans ~8.4 kb; it consists of 13 exons (GenBank accession number Z66539) and encodes a protein of 635 amino acids with a predicted molecular weight of 70 kDa (Sandoval et al. 1996) (fig. 1).

The use of proton magnetic resonance spectroscopy (H-MRS) resulted in the identification of three inborn errors of metabolism: two creatine biosynthesis errors (AGAT deficiency [MIM 602360] and GAMT deficiency

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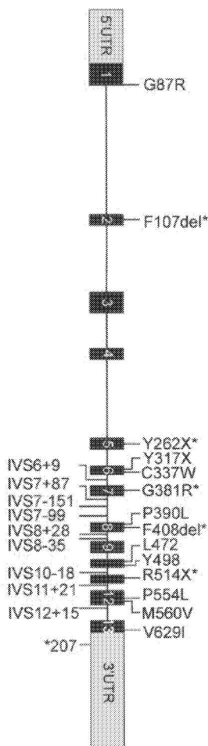

Figure 1 Mutations and polymorphisms in patients with XLMR. Primer sequences were designed specifically to amplify all exons of the *SLC6A8* gene (and not the *SLC6A10* gene, a presumed creatine transporter pseudogene mapped at chromosome 16 [Eichler et al. 1996]), including short fragments of the flanking intronic sequences. By comparing the *SLC6A8* and *SLC6A10* sequences, we have found that at least two nucleotide variations were present in all amplicons, confirming selective amplification of the *SLC6A8* sequences (data not shown). PCR reactions were performed with HotStar *Taq* (Qiagen) in a PE Applied Biosystems model 9700. Direct sequence analysis was performed on purified PCR products (Millipore vacufold) by use of BigDye v3.1 terminators and an ABI 3100 sequence machine (PE Applied Biosystems). The obtained electropherograms were assembled and analyzed to identify potential genomic alterations by use of the Mutation Surveyor software package (SoftGenetics). Sequence variants were annotated according the guidelines of den Dunnen and Antonarakis (2001). On the basis of impaired uptake in fibroblasts, five alterations (*asterisks [*]*) have been proven elsewhere to be mutations. p.F107del was also found in our XLMR cohort. One novel nonsense mutation (p.Y317X) is strongly predictive of impaired creatine uptake. p.M560V is a rare polymorphism, and p.V629I is an unclassified variant. The implications of the translational silent variant, the IVS variant, and the 3′ UTR variant could not yet be investigated, and these variants are therefore assigned as "unclassified." IVS variants in introns 1, 2, 3, and 4 may have been missed, since only small exon-flanking parts were included.

[MIM 601240]) and one creatine transport error (SLC6A8 deficiency [MIM 300036]) (Stockler et al. 1994, 1996*a*, 1996*b*; Schulze et al. 1997; Bianchi et al. 2000; van der Knaap et al. 2000; Cecil et al. 2001; Item et al. 2001; Salomons et al. 2001; Bizzi et al. 2002; deGrauw et al. 2002; Hahn et al. 2002; Salomons et al. 2003). These creatine-deficiency syndromes (CDS) share the almost-complete lack of creatine/phosphocreatine in the brain, as measured by in vivo H-MRS. Metabolite measurements in urine and plasma are indicative of the specific disorders (e.g., in SLC6A8-deficient males, the creatine:creatinine [Cr:Crn] ratio is increased in urine, and guanidinoacetate is increased in urine and plasma of GAMT-deficient patients). Characteristic in the clinical presentation of all CDS are mental retardation, expressive speech and language delay, and epilepsy (varying from intractable seizures in GAMT-deficient patients to mild epileptic or febrile seizures in AGAT-deficient and transporter-deficient patients). GAMT-deficient patients and transporter-deficient patients may show autistic behavior; in GAMT-deficient patients with a severe phenotype, (extra)pyramidal symptoms are present (Salomons et al. 2003; Stromberger et al. 2003). Female carriers of *SLC6A8* mutations may have learning disabilities and/or behavioral problems.

In Western countries, mental retardation (MR) affects 2%–3% of the general population (Chelly and Mandel 2001). For the majority of the cases of inherited MR, the genetic cause has not yet been elucidated. The larger number of males than females in the MR population (~70%) suggests a high contribution of X-linked disorders. X-linked mental retardation (XLMR) is estimated to account for 5%–12% of all cases of MR, with a relatively large (2%) contribution of fragile-X syndrome (fraX) (Herbst and Miller 1980; de Vries et al. 1997). The fact that >15 SLC6A8-deficient families (five families in one metropolitan area) have been identified in the 2 years after the first recognition of this disorder suggests a high incidence of creatine transporter deficiency in the Western population. Two prominent features of SLC6A8 deficiency are MR and X-linked inheritance of the disease. We therefore investigated by DNA sequence analysis the prevalence of *SLC6A8* defects in a panel of 290 unrelated families of the European XLMR Consortium (European XLMR Consortium Web site). Families are included in this panel if at least two males in the family are affected. Informed consent has been obtained from the parents or caretakers of the affected males. Of the 290 cases, 2 were excluded from this study, since we were unable to amplify, by PCR, DNA sequences of the *SLC6A8* gene and of two autosomal genes, because of the poor quality of the DNA. In addition, DNAs of 276 healthy controls were similarly analyzed.

In the European XLMR panel, seven novel nucleotide

substitutions in the ORF of *SLC6A8* were encountered: six missense variations/mutations and one insertion resulting in an immediate nonsense codon. In addition, one known pathogenic single–amino acid deletion was found. Furthermore, one translational silent change, eight intervening sequence (IVS) variants, and one substitution in the 3′ UTR were detected—none of which were found in either the 276 controls or the human EST database (table 1; fig. 1).

In DNA of the index patient of family T115, a deletion of 3 bp (c.319_321delCTT) in *SLC6A8* was detected, which results in the loss of phenylalanine at position p.107 (p.F107del). The mutation is located in a short repeat of two phenylalanines in exon 2. This repeat is part of transmembrane-spanning domain (TM) II, which is highly conserved among all known creatine transporters (i.e., bovine, rat, rabbit, marbled electric ray, and mouse [see fig. 2]) and within the Na^{+}- and Cl^{-}-dependent neurotransmitter transporter family. The mutation segregates within the family (fig. 3*A*). *SLC6A8* F107del was previously reported in an unrelated family (deGrauw et al. 2002) (fig. 1). The index male of that family showed impairment of creatine uptake in fibroblasts. The following observations indicate that p.F107del is pathogenic: (1) p.F107 is highly conserved between species; (2) p.F107 is located in a conserved area within its solute carrier family; (3) F107del segregates with the XLMR phenotype; (4) the mutation was detected in an unrelated family with SLC6A8 deficiency; (5) urine of affected males with this mutation showed an increased Cr:Crn ratio, a hallmark of this disease; and (6) fibroblasts with this deletion are impaired in creatine uptake.

In DNA of the index patient of family P66, an insertion of adenosine at position c.951 in exon 6 (c.950_951insA) of the *SLC6A8* gene was found. This mutation predicts a premature stop (p.Y317X) that results in a truncated protein of 316 amino acids that lacks 319 amino acids of the C-terminus, including the putative TM VII–XII of SLC6A8 (fig. 2). The mutation most likely results in an unstable and/or inappropriately folded protein that is completely inactive. Indeed, two other nonsense mutations in the *SLC6A8* gene (p.R514X and p.Y262X) (fig. 1) have been shown to impair creatine uptake and are pathogenic.

In DNA of the index patient of family T31, the transition G→A at position c.259 in the *SLC6A8* gene was found. This transition results in the substitution p.G87R. The mutation is located in a short repeat of three glycines in exon 1. This repeat is located between the putative TM I and II in a small intracellular loop that is highly conserved among all known creatine transporters and within the neurotransmitter transporter family SLC6 (fig. 2). The mutation results in the substitution of the nonpolar and neutral glycine for the polar and basic arginine. The highly conserved nature of p.G87 and the clear difference in the chemical properties of the wild-type and mutant amino acids indicate that the p.G87R mutation is pathogenic. Because no additional biological

Table 1

Nucleotide Changes in *SLC6A8* in the XLMR Panel

Genomic Mutation	cDNA Mutation	Deduced Effect	Family
g.2933G→A	c.259G→A	p.G87R[a]	T31
g.4533_4535delCTT	c.319_321delCTT	p.F107del[a]	T115
g.7400_7401insA	c.950_951insA	p.Y317stop[a]	P66
g.7461C→G	c.1011C→G	p.C337W[a]	N87
g.8032C→T	c.1169C→T	p.P390L[a]	T132
g.8883C→T	c.1661C→T	p.P554L[a]	D11
g.8900A→G	c.1678A→G	p.M560V[b]	P18
g.9291G→A	c.1885G→A	p.V629I[c]	N67
g.8467G→A	c.1416G→A	p.I472[c]	⩾1 family
IVS6+9C→T	ND	ND	⩾1 family
IVS7-151_152delGA	ND	ND	⩾1 family
IVS7-99C→A	ND	ND	⩾1 family
IVS8+28C→T	ND	ND	⩾1 family
IVS8-35G→A	ND	ND	⩾1 family
IVS10-18C→T	ND	ND	⩾1 family
IVS11+21G→A	ND	ND	⩾1 family
IVS12+15C→T	ND	ND	⩾1 family
*207G→C	ND	ND	⩾1 family
IVS12+32C→A	ND	ND	⩾1 family

NOTE.—The *SLC6A8* nucleotide changes shown in this table were not found in the 276 controls or in the human EST database. ND = not determined.

[a] Pathogenic mutation.

[b] Polymorphism.

[c] Unclassified variant.

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                                                                                                          ♦
A8    1   ---------------MAKKSAE-NGIY--SVSGDEKKGPLIAPGPDGAPAKGDGPVGLGT------PGGR----------LAVPPRETWTRQMDFIMSCV
RN    1   ---------------MAKKSAE-NGIY--SVSGDEKKGPLIVSGPDGAPSKGDGPAGLGA------PSSR----------LAVPPRETWTRQMDFIMSCV
MM    1   ---------------MAKKSAE-NGIY--SVSGDEKKGPLIVSGPDGAPAKGDGPAGLGA------PGGR----------LAVPPRETWTRQMDFIMSCV
BT    1   ---------------MANKSTE-NGIY--SVSGEEKKGPLIAPGPDGAPAKGDGPAALGA------PGSL----------LAVPPRETWTRQMDFIMSCV
OC    1   ---------------MAKKSAE-NGIY--SVSGDEKKGPLIAPGPDGAPAKGDGPAGLGA------PGGC----------LAVPPRETWTRQMDFIMSCV
TM    1   ---------------MPSRAVR-R-CP--GHLCKEMRAPRRAQPPD---------VPAGE------PGS----------------RVTWSRQMDFIMSCV
A1    1   ---------------MATNGSK-VADG--QISTEVSEAPVANDKPK--------TLVVKV------QKKA----------ADLPDRDTWKGRFDFLMSCV
A2    1   ----MLLARMNPQVQPENNGAD-TGPE--QPLRARKTAELLVVKERNG-VQC----LLAP------RDG-------------DAQPRETWGKKIDFLLSVV
A3    1   ------MSKSKCSVGLMSSVVA-PAKE--PNAVGPKEVELILVKEQNG-VQLTSSTLTNP------RQSP----------VEAQDRETWGKKIDFLLSVI
A4    1   ----METTPLNSQKQLSACEDG-EDCQ--ENGVLQKVVPTPGDKVESGQISNGYSAVPSPGAGDDTRHSIPATTTTLVAELHQGERETWGKKVDFLLSVI
A5    1   ----MDCSAPKEMNKLPANSPEAPPPRLRDLREAQGAQASPPPGSSGPGPEGDANVSVGKGTLENMSQTSTVVLGTDGITGDENKARGNWSSKIDFIISMV
A6    1   ---------------MATKEKL-Q-CL--KDFHKDMVKPSPGKSPG--------TRPEDE------AEG-----------KPPQREKWSSKIDFVLSVA
A7    1   ------------MKKLQGAHLRKPVTP--DLLMTPSDQGD---------------VDLDV-------------------DFAAHRGNWTGKLDFLLSCI
A9    1   ---------------MAAAHG--PVAP--SSPEQNGAVPS---------------EATKR-------------------DQNLKRGNWGNQIEFVLTSV
A11   1   MTAEKALPLGNGKAAEEARESE-A-PG--GGCSSGGAAPA-----R--------HPRVKR------DK-------------AVHERGHWNNKVEFVLSVA
A12   1   ---------------MDGKVAV-Q-ER--GPPAVSWVPEE-----G--------EKLDQE------DED------------QVKDRGQWTNKMEFVLSVA
A13   1   ---------------MDSRVS--------GTTSNGETKPV-----Y--------PVMEKK------EED------------GTLERGHWNNKMEFVLSVA
A14   1   ------------MDKLKC-----PS--FFKCREKEKVSAS----S-----E---NFHVGE-------------------NDENQDRGNWSKKSDYLLSMI

                                                                                   ┌Cys144
           ————TMI————             ————TMII————                            ————TMIII————
A8    67  GFAVGLGNVWRFPYLCYKNGGGVFLIPYVLIALVGGIPIFFLEISLGQFMKAGSINVWN-ICPLFKGLGYASMVIVFYCNTYYIMVLAWGFYYLVKSFTT
RN    67  GFAVGLGNVWRFPYLCYKNGGGVFLIPYVLIALVGGIPIFFLEISLGQFMKAGSINVWN-ICPLFKGLGYASMVIVFYCNTYYIMVLAWGFYYLVKSFTT
MM    67  GFAVGLGNVWRFPYLCYKNGGGVFLIPYVLIALVGGIPIFFLEISLGQFMKAGSINVWN-ICPLFKGLGYASMVIVFYCNTYYIMVLAWGFYYLVKSFTT
BT    67  GFAVGLGNVWRFPYLCYKNGGGVFLIPYILIALIGGIPIFFLEISLGQFMKAGSINVWN-ICPLFKGLGYASMVIVFYCNTYYIMVLAWGFYYLVKSFTT
OC    67  GFAVGLGNVWRFPYLCYKNGGGVFLIPYVLIALVGGIPIFFLEISLGQFMKAGSINVWN-ICPLFKGLGYASMVIVFYCNTYYIMVLAWGFYYLVKSFTT
TM    51  GFAVGLGNVWRFPYLCYKNGGGVFLIPYLLVAVFGGIPIFFLEISLGQFMKAGGINAWN-IAPLFKGLGYASMVIVFFCNTYYILVLTWSSFYLVQSFSS
A1    59  GYAIGLGNVWRFPYLCGKNGGGAFLIPYFLTLIFAGVPLFLLECSLGQYTSIGGLGVWK-LAPMFKGVGLAAAVLSFWLNIYYIVIISWAIYYLYNSFTT
A2    71  GFAVDLANVWRFPYLCYKNGGGAFLIPYTLFLIIAGMPLFYMELALGQYNREGAATVW-KICPFFKGVGYAVILIALYVGFYYNVIIAWSLYYLFSSFTL
A3    75  GFAVDLANVWRFPYLCYKNGGGAFLVPYLLFMVIAGMPLFYMELALGQFNREGAAGVW-KICPILKGVGFTVILISLYVGFFYNVIIAWALHYLFSSFTT
A4    94  GYAVDLGNVWRFPYICYQNGGGAFLLPYTIMAIFGGIPLFYMELALGQYHRNGCISIWRKICPIFKGIGYAICIIAFYIASYYNTIMAWALYYLISSFTD
A5   206  GYAVGLGNVWRFPYLAFQNGGGAFLIPYLMMLALAGLPIFFLEVSLGQFASQGPVSVWK-AIPALQGCGIAMLIISVLIAIYYNVIICYTLFYLFASFVS
A6    56  GGFVGLGNVWRFPYLCYKNGGGAFLIPYFIFLFGSGLPVFFLEIIIGQYTSEGGITCWEKICPLFSGIGYASVVIVSLLNVYYIVILAWATYYLFQSFQK
A7    52  GYCVGLGNVWRFPYRAYTNGGGAFLVPYFLMLAICGIPLFFLELSLGQFSSLGPLAVWK-ISPLFKGAGAAMLLIVGLVAIYYNMIIAYVLFYLFASLTS
A9    47  GYAVGLGNVWRFPYLCYRNGGGAFMFPYFIMLIFCGIPLFFMELSFGQFASQGCLGVWR-ISPMFKGVGYGMMVVSTYIGIYYNVVICIAFYYFFSSMTH
A11   65  GEIIGLGNVWRFPYLCYKNGGGAFLIPYVVFFICCGIPVFFLETALGQFTSEGGITCWRKVCPLFEGIGYATQVIEAHLNVYYIIILAWAIFYLSNCFTT
A12   51  GEIIGLGNVWRFPYLCYKNGGGAFFIPYFIFFFVCGIPVFFLEVALGQYTSQGSVTAWRKICPLFQGIGLASVVIESYLNVYYIIILAWALFYLFSSFTS
A13   47  GEIIGLGNVWRFPYLCYKNGGGAFFIPYLVFLFTCGIPVFLLETALGQYTSQGGVTAWRKICPIFEGIGYASQMIVILLNVYYIIVLAWALFYLFSSFTI
A14   51  GYAVGLGNVWRFPYLTYSNGGGAFLIPYAIMLALAGLPLFFLECSLGQFASLGPVSVWR-ILPLFQGVGITMVLISIFVTIYYNVIIAYSLYYMFASFQS
                                G87R                └F107del

                                        ▲     ▲
A8   166  TLPWATCGHTWNTPDCVEI----FRHEDCANASLANLTCDQLADRR-------------------SPVIEFWENKVLRLS--GGLEVPGALNWEVTLCLLA
RN   166  TLPWATCGHTWNTPDCVEI----FRHEDCANASLANLTCDQLADRR-------------------SPVIEFWENKVLRLS--TGLEVPGALNWEVTLCLLA
MM   166  TLPWATCGHTWNTPDCVEI----FRHEDCANASLANLTCDQLADRR-------------------SPVIEFWENKVLRLS--TGLEVPGALNWEVTLCLLA
BT   166  TLPWATCGHTWNTPDCVEI----FRHEDCANATMANLTCDQLADRR-------------------SPVIEFWENKVLRLS--EGLEVPGALNWEVTLCLLT
OC   166  TLPWATCGHTWNTPDCVEI----FRHEDCANGSLANLTCDQLAERR-------------------SPVIEFWENKVLRLS--GGLEVPGALNSEVTLCLLA
TM   150  PLPWASCNNTWNTAACYEA----G-----ANASTE--IYPPTAPAQ-------------------SSIVQFWERRVLRLS--SGLGDVGEIGWELTLCLTA
A1   158  TLPWKQCDNPWNTDRCFS-------------NYS-----MVNTTNMT------------------SAVVEFWERNMHQMT--DGLDKPGQIRWPLAITLAI
A2   170  NLPWTDCGHTWNSPNCTDP----KL----LNGSVLGNHTKYSKYKF-------------------TPAAEFYERGVLHLHESSGIHDIGLPQWQLLLCLMV
A3   174  ELPWIHCNNSWNSPNCSDA----HP----GD-SSGDSSGLNDTFGT-------------------TPAAEYFERGVLHLHQSHGIDDLGPPRWQLTACLVL
A4   194  QLPWTSCKNSWNTGNCTNY----FS----ED------NITWTLHST-------------------SPAEEFYTRHVLQIHRSKGLQDLGGISWQLALCIML
A5   305  VLPWGSCNNPWNTPECKDKTKLLLDSCVISDHPKIQIKNSTFCMTAYPNVTMVNFTSQANKTFVSGSEEYFKYFVLKI--SAGIEYPGEIRWPLALCLFL
A6   156  ELPWAHCNHSWNTPHCMED----TMR---KNKSVW--ITISSTNFT-------------------SPVIEFWERNVLSLS--PGIDHPGSLKWDLALCLLL
A7   151  DLPWEHCGNWWNTELC-------LEHRVSKDGN--GALPLNLTCTV-------------------SPSEEYWSRYVLHIQGSQGIGSPGEIRWNLCLCLLL
A9   146  VLPWAYCNNPWNTHDCAG----VLDASNLTNGSRPAALPSNLSHLLNHSLQRT------------SPSEEYWRLYVLKL--SDDIGNFGEVRLPLLGCLGV
A11  165  ELPWATCGHEWNTENCVE-----FQK---LNVSNY--SHVSLQNAT-------------------SPVMEFWEHRVLAIS--DGIEHIGNLRWELALCLLA
A12  151  ELPWTTCNNFWNTEHCTD-----FLN---HSGAG---TVTPFENFT-------------------SPVMEFWERRVLGIT--SGIHDLGSLRWELALCLLL
A13  147  DLPWGGCYHEWNTEHCME-----FQK---TNGS----LNGTSENAT-------------------SPVIEFWERRVLKIS--DGIQHLGALRWELALCLLL
A14  150  ELPWKNCSS-WSDKNCSRSPIVTHCNVSTVNKGIQEIIQMNKSWVDINNFTCIN-GSEIYQPGQLPSEQYWNKVALQR--SSGMNETGVIVWYLALCLLL

           —TMIV———      ♦     ————TMV————          ■       ■        ■      ■        ————TMVI————
A8   242  CWVLVYFCVWKGVKSTGKIVYFTATFPYVVLVVLLVRGVLLPGALDGIIYYLKPD--WSKLGSPQVWIDAGTQIFFSYAIGLGALTALGSYNRFNNNCYK
RN   242  CWVLVYFCVWKGVKSTGKIVYFTATFPYVVLVVLLVRGVLLPGALDGIIYYLKPD--WSKLGSPQVWIDAGTQIFFSYAIGLGALTALGSYNRFNNNCYK
MM   242  CWVLVYFCVWKGVKSTGKIVYFTATFPYVVLVVLLVRGVLLPGALDGIIYYLKPD--WSKLGSPQVWIDAGTQIFFSYAIGLGALTALGSYNRFNNNCYK
BT   242  CWVLVYFCVWKGVKSTGKIVYFTATFPYVVLVVLLVRGVLLPGALDGIIYYLKPD--WSKLASPQVWIDAGTQIFFSYAIGLGALTALGSYNRFNNNCYK
OC   242  CWVLVYFCVWKGVKSTGKIVYFTATFPYVVLVVLLVRGVLLPGALDGIIYYLKPD--WSKLRSPQVWIDAGTQIFFSYAIGLGALTALGSYNRFNNNCYK
TM   219  TWMLVYFCIWKGVKTSGKVVYVTATFPYIILVILLVRGVTLHGAVQGIVYYLQPD--WGKLGEAQVWIDAGTQIFFSYAIGLGTLTALGSYNQLHNDCYK
A1   221  AWILVYFCIWKGVGWTGKVVYFSATYPYIMLIILFFRGVTLPGAKEGILFYITPN--FRKLSDSEVWLDAATQIFFSYGLGLGSLIALGSYNSFHNNVYR
A2   244  VVIVLYFSLWKGVKTSGKVVWITATLPYFVLFVLLVHGVTLPGASNGINAYLHID--FYRLKEATVWIDAATQIFFSLGAGFGVLIAFASYNKFDNNCYR
A3   247  VIVLLYFSLWKGVKTSGKVVWITATMPYVVLTALLLRGVTLPGAIDGIRAYLSVD--FYRLCEASVWIDAATQVCFSLGVGFGVLIAFSSYNKFTNNCYR
A4   262  IFTVIYFSIWKGVKTSGKVVWVTATFPYIILSVLLVRGATLPGAWRGVLFYLKPN--WQKLLETGVWIDAAAQIFFSLGPGFGVLLAFASYNKFNNNCYQ
A5   403  AWVIVYASLAKGIKTSGKVVYFTATFPYVVLVILLIRGVTLPGAGAGIWYFITPK--WEKLTDATVWKDAATQIFFSLSAAWGGLITLSSYNKFHNNCYR
A6   227  VWLVCFFCIWKGVRSTGKVVYFTATFPFAMLLVLLVRGLTLPGAGRGIKFYLYPD--ITRLEDPQVWIDAGTQIFFSYAICLGAMTSLGSYNKYKYNSYR
A7   224  AWVIVFLCILKGVKSSGKVVYFTATFPYLILLMLLVRGVTLPGAWKGIQFYLTPQ--FHHLLSSKVWIEAALQIFYSLGVGFGGLLTFASYNTFHQNIYR
A9   229  SWLVVFLCLIRGVKSSGKVVYFTATFPYVVLTILFVRGVTLEGAFDGIMYYLTPQ--WDKILEAKVWGDAASQIFYSLACAWGGLITMASYNKFHNNCYR
A11  235  AWTICYFCIWKGTKSTGKVVYVTATFPYIMLLILLIRGVTLPGASEGIKFYLYPD--LSRLSDPQVWVDAGTQIFFSYAICLGCLTALGSYNNYNNNCYR
A12  220  AWVICYFCIWKGVKSTGKVVYFTATFPYLMLVILLIRGVTLPGAYQGIIYYLKPD--LFRLKDPQVWMDAGTQIFFSFAICQGCLTALGSYNKYHNNCYK
A13  215  AWVICYFCIWKGVKSTGKVVYFTATFPYLMLVILLIRGVTLPGAAQGIQFYLYPN--LTRLWDPQVWMDAGTQIFFSFAICLGCLTALGSYNKYHNNCYR
A14  246  AWLIVGAALFKGIKSSGKVVYFTALFPYVVLLILLVRGATLEGASKGISYYIGAQSNFTKLKEAEVWKDAATQIFYSLSVAWGGLVALSSYNKFKNNCFS
                               Y262X                                                  Y317X           C337W
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Figure 2 Multiple-sequence alignment with hierarchical clustering among the SLC6A8 proteins of different species and the superfamily of neurotransmitter transporters. The SLC6A8 protein sequence is shown for the following species: *Homo sapiens* (A8), *Rattus norvegicus* (RN), *Mus musculus* (MM), *Bos taurus* (BT), *Oryctolagus cuniculus* (OC), and *Torpedo marmorata* (TM). *SLC6A8* genes were identified by a protein blast (BLASTP). Alignment was determined by the ClustalW program; the SLC6A8 proteins that were used for this analysis were the ones identified by the BLASTP search to be most related to the *Homo sapiens* SLC6A8 protein. All were saved in FASTA format. The BOXSHADE program was used to visualize identical amino acids (*highlighted in black*) and functionally conserved amino acids (*gray*). Functionally conserved amino acids are classified as follows: V, I, L, and M; D, E, Q, and N; F, Y, and W; G, S, T, P, and A; and K, R, and H. The codes A1–A14 represent neurotransmitter transporters SLC6A1–SLC6A14 (A1 = GABA1, A2 = noradrenaline, A3 = dopamine, A4 = serotonine, A5 = glycine2, A6 = taurine, A7 = proline, A9 = glycine 1, A11 = GABA3, A12 = betaine/GABA, A13 = GABA2, and A14 = ATB^{0+}). In the protein sequence of A5, certain amino acids (20–46, 51–93, 116–127, 143–152, and 168–184) were cut out, because these stretches occur only in SLC6A5 and would make the figure disorganized. TMI–TMXII = putative transmembrane domain, predicted by ExPASy (Swiss-Prot S6A8_Human P48029); ♦ = putative cAMP-PK phosphorylation site (2 ×); ▲ = putative N-glycosylation sites (3 ×); ■ = putative Leu zipper motif (4 ×).

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                 ---------TMVII----------                                              ---------TMVIII-------
A8   340 DAIILALINSGTSFFAGFVVFSILGFMAAEQGVHISKVAES-GPGLAFIAYPRAVTLMPVAPLWAALFFFMLLLLGLDSQFVGVEGFITGLLDLLP-ASY
RN   340 DAIILALINSGTSFFAGFVVFSILGFMATEQGVHISKVAES-GPGLAFIAYPRAVTLMPVAPLWAALFFFMLLLLGLDSQFVGVEGFITGLLDLLP-ASY
MM   340 DAIILALINSGTSFFAGFVVFSILGFMATEQGVHISKVAES-GPGLAFIAYPRAVTLMPVAPLWAALFFFMLLLLGLDSQFVGVEGFITGLLDLLP-ASY
BT   340 DAIILALINSGTSFFAGFVVFSILGFMATEQGVHISKVAES-GPGLAFIAYPRAVTLMPVAPLWAALFFFMLLLLGLDSQFVGVEGFITGLLDLLP-ASY
OC   340 DAIILALINSGTSFFAGFVVFSILGFMATEQGVHISKVAES-GPGLAFIAYPRAVTLMPVAPLWAALFFFMLLLLGLDSQFVGVEGFITGLLDLLP-ASY
TM   317 DAFILSLVNSATSFFAGLVVFSILGFMAVEEGVDISVVAES-GPGLAFIAYPKAVTLMPFPQVWAVLFFIMLLCLGLGSQFVGVEGFVTAILDLWP-SKF
A1   319 DSIIVCCINSCTSMFAGFVIFSIVGFMAHVTKRSIADVAAS-GPGLAFLAYPEAVTQLPISPLWAILFFSMLLMLGIDSQFCTVEGFITALVDEYP-RLL
A2   342 DALLTSSINCITSFVSGFAIFSILGYMAHEHKVNIEDVATE-GAGLVFILYPEAISTLSGSTFWAVVFFVMLLALGLDSSMGGMEAVITGLADDF--QVL
A3   345 DAIVTTSINSLTSFSSGFVVFSFLGYMAQKHSVPIGDVAKD-GPGLIFIIYPEAIATLPLSSAWAVVFFIMLLTLGIDSAMGGMESVITGLIDEF--QLL
A4   360 DALVTSVVNCMTSFVSGFVIFTVLGYMAEMRNEDVSEVAKDAGPSLLFITYAEAIANMPASTFFAIIFFLMLITLGLDSTFAGLEGVITAVLDEFP-HVW
A5   501 DTLIVTCTNSATSIFAGFVIFSVIGFMANERKVNIENVADQ-GPGIAFVVYPEALTRLPLSPFWAIIFFLMLLTLGLDTMFATIETIVTSISDEFP-KYL
A6   325 DCMLLGCLNSGTSFVSGFAIFSILGFMAQEQGVDIADVAES-GPGLAFIAYPKAVTMMPLPTFWSILFFIMLLLLGLDSQFVEVEGQITSLVDLYP-SFL
A7   322 DTFIVTLGNAITSILAGFAIFSVLGYMSQELGVPVDQVAKA-GPGLAFVVYPQAMTMLPLSPFWSFLFFFMLLTLGLDSQFAFLETIVTAVTDEFP-YYL
A9   327 DSVIISITNCATSVYAGFVIFSILGFMANHLGVDVSRVADH-GPGLAFVAYPEALTLLPISPLWSLLFFFMLILLGLGTQFCLLETLVTAIVDEVGNEWI
A11  333 DCIMLCCLNSGTSFVAGFAIFSVLGFMAYEQGVPIAEVAES-GPGLAFIAYPKAVTMMPLSPLWATLFFMMLIFLGLDSQFVCVESLVTAVVDMYP-KVF
A12  318 DCIALCFLNSATSFVAGFVVFSILGFMSQEQGVPISEVAES-GPGLAFIAFPKAVTMMPLSQLWSCLFFIMLIFLGLDSQFVCVECLVTASIDMFP-RQL
A13  313 DCIALCFINSGTSFVAGFAIFSILGFMSQEQGVPISEVAES-GPGLAFIAYPRAVVMLPFSPLWACCFFFMVVLLGLDSQFVCVESLVTALVDMYP-HVF
A14  346 DAIVVCLTNCLTSVFAGFAIFSILGHMAHISGKEVSQVVKS-GFDLAFIAYPEALAQLPGGPFWSILFFFMLLTLGLDSQFASIETITTTIQDLFP-KVM
                                                  G381R    P390L                └F408del

                ---------TMIX----------                      ---------TMX----------                     ------------
A8   438 YFRFQREISVALCCALCFVIDLSMVTD-----GGMYVFQLFDYYSASGTTLLWQAFWECVVVAWVYGADRFMDDIACMIGYRPCPW---MKWCWSFFTPL
RN   438 YFRFQREISVALCCALCFVIDLSMVTD-----GGMYVFQLFDYYSASGTTLLWQAFWECVVVAWVYGADRFMDDIACMIGYRPCPW---MKWCWSFFTPL
MM   438 YFRFQREISVALCCALCFVIDLSMVTDVSGGKGGMYVFQLFDYYSASGTTLLWQAFWECVVVAWVYGADRFMDDIACMIGYRPCPW---MKWCWSFFTPL
BT   438 YFRFQREISVALCCTICFVIDLSMVTD-----GGMYVFQLFDYYSASGTTLLWQAFWECVVVAWVYGADRFMDDVACMIGYRPCPW---MKWCWSFFTPL
OC   438 YFRFQREISVALCCALCFVIDLSMVQM-----AGMYVFQLFDYYSASGTTLLWQAFWECVAVAWVYGADRFMDDIACMIGYRPCPW---MKWCWSFFTPL
TM   415 SFRYLREVVVAMVICLSFLIDLSMITE-----GGMYIFQIFDYYSASGTTLLWTAFWECVAVAWVYGGDRYLDDLAWMLGYRPWAL---VKWCWSVITPL
A1   417 R--NRRELFIAAVCIISYLIGLSNITQ-----GGIYVFKLFDYYSASGMSLLFLVFFECVSISWFYGVNRFYDNIQEMVGSRPCIW---WKLCWSFFTPI
A2   439 KR--HRKLFTFGVTFSTFLLALFCITK-----GGIYVLTLLDTFAA-GTSILFAVLMEAIGVSWFYGVDRFSNDIQQMMGFRPGLY---WRLCWKFVSPA
A3   442 HR--HRELFTLFIVLATFLLSLFCVTN-----GGIYVFTLLDHFAA-GTSILFGVLIEAIGVAWFYGVGQFSDDIQQMTGQRPSLY---WRLCWKLVSPC
A4   459 AK--RRERFVLAVVITCFFGSLVTLTF-----GGAYVVKLLEEYAT-GPAVLTVALIEAVAVSWFYGITQFCRDVKEMLGFSPGWF---WRICWVAISPL
A5   599 RT--HKPVFTLGCCICFFIMGFPMITQ-----GGIYMFQLVDTYAA-SYALVIIAIFELVGISYVYGLQRFCEDIEMMIGFQPNIF---WKVCWAFVTPT
A6   423 RKGYRREIFIAFVCSISYLLGLTMVTE-----GGMYVFQLFDYYAASGVCLLWVAFFECFVIAWIYGGDNLYDGIEDMIGYRPGPW---MKYSWVIT-PV
A7   420 RP--KKAVFSGLICVAMYLMGLILTTD-----GGMYWLVLLDDYSA-SFGLMVVVITTCLAVTRVYGIQRFCRDIHMMLGFKPGLY---FRACWLFLSPA
A9   426 LQ--KKTYVTLGVAVAGFLLGIPLTSQ-----AGIYWLLLMDNYAA-SFSLVVISCIMCVAIMYIYGHRNYFQDIQMMLGFPPPLF---FQICWRFVSPA
A11  431 RRGYRRELLILALSVISYFLGLVMLTE-----GGMYIFQLFDSYAASGMCLLFVAIFECICIGWVYGSNRFYDNIEDMIGYRPPSL---IKWCWMIMTPG
A12  416 RKSGRRELLILTIAVMCYLIGLFLVTE-----GGMYIFQLFDYYASSGICLLFLSLFEVVCISWVYGADRFYDNIEDMIGYRPWPL---VKISWLFLTPG
A13  411 RKKNRREVLILGVSVVSFLVGLIMLTE-----GGMYVFQLFDYYAASGMCLLFVAIFESLCVAWVYGAKRFYDNIEDMIGYRPWPL---IKYCWLFLTPA
A14  444 KK--MRVPITLGCCLVLFLLGLVCVTQ-----AGIYWVHLIDHFCA-GWGILIAAILELVGIIWIYGGNRFIEDTEMMIGAKRWIFWLWWRACWFVITPI
                                                                                       R514X

   --TMXI-----       ▲         ---------TMXII---------
A8   530 VCMGIFIFNVVYYEPLVYNNTYVYPWWGEAMGWAFALSSMLCVPLHLLGCLLRAKG-TMAERWQHLTQPIWGLHHLEYRAQDADVRG----LTTLTP---
RN   530 VCMGIFIFNVVYYKPLVYNNTYVYPWWGEAMGWAFALSSMLCVPLHLLGCLLRAKG-TMAERWQHLTQPIWGLHHLEYRAQDADVRG----LTTLTP---
MM   535 VCMGIFIFNIVYYEPLVYNNTYVYPWWGEAMGWAFALSSMLCVPLHLLGCLLRAKG-TMAERWQHLTQPIWGLHHLEYRAQDADVRG----LTTLTP---
BT   530 VCMGIFIFNVVYHEPLVYNNTYVYPWWGEAVGWAFALSSMLCVPLHLLGCLLRAKG-TMAERWQHLTQPIWGLHHLEYRAQDSDVRG----LTTLTP---
OC   530 VCMGIFIFNIVYYKPLVYNKTYVYPWWGEAMGWAFALSSMLCVPLHLLGCLLRAKG-TMAERWQHLTQPVWGLHHLEYRAQDADVRG----LTTLTP---
TM   507 VCMGIFTFHLVNYKPLTYNKTYTYPWWGEAIGWCLALASMLCVPTTVLYSLSRGRG-SLKERWRKLTTPVWASHHLAYKMAGAKIN-----QPCEGV---
A1   507 IVAGVFIFSAVQMTPLTMGN-YVFPKWGQGVGWLMALSSMVLIPGYMAYMFLALKG-SLKQRIQVMVQPSEDTVRPENGPEH-----------------
A2   528 FLLFVVVVSIINFKPLTYDD-YIFPPWANWVGWGIALSSMVLVPIYVIYKFLSTQG-SLWERLAYGITPENEHHLVAQRDIR-----------------
A3   531 FLLFVVVVSIVTFRPPHYGA-YIFPDWANALGWVIATSSMAMVPIYAAYKFCSLPG-SFREKLAYAIAPEKDRELVDRGEVR-----------------
A4   548 FLLFIICSFLMSPPQLRLFQ-YNYPYWSIILGYCIGTSSFICIPTYIAYRLIITPG-TFKERIIKSITPETPTEIP-CGDIR-----------------
A5   688 ILTFILCFSFYQWEPMTYGS-YRYPNWSMVLGWLMLACSVIWIPIMFVIKMHLAPG-RFIERLKLVCSPQPDWGPFLAQHRGERYKNMIDPLGT------
A6   514 LCVGCFIFSLVKYVPLTYNKTYVSPTWAIGLGWSLALSSMLCVPLVIVIRLCQTEG-PFLVRVKYLLTPREPNRWAVEREGA----------TPYNS---
A7   509 TLLALLVYSIVKYQPSEYGS-YRFPPWAELLGILMGLLSCLMIPAGMLVAVLREEG-SLWERLQQASRPAMDWGPSLEENRTGMYVATLAGSQSPKPLMV
A9   515 IIFFILVFTVIQYQPITYNH-YQYPGWAVAIGFLMALSSVLCIPLYAMFRLCRTDGDTLLQRLKNATKPSRDWGPALLEHRTGRYAPTIAPSPE-DGFEV
A11  523 ICAGIFIFFLIKYKPLKYNNIYTYPAWGYGIGWLMALSSMLCIPLWICITVWKTEG-TLPEKLQKLTTPSTDLKMRGKLGVSPRMVTVNDCDAKLKS---
A12  508 LCLATFLFSLSKYTPLKYNNVYVYPPWGYSIGWFLALSSMVCVPLFVVITLLKTRG-PFRKRLRHVITPDSSLPQPKQHPCLDGSAGRN---FGPSP---
A13  503 VCTATFLFSLIKYTPLTYNKKYTYPWWGDALGWLLALSSMVCIPAWSLYRLGTLKG-PFRERIRQLMCPAEDLPQRNPAGPS----------APATP---
A14  536 LLIAIFIWSLVQFHRPNYGA-IPYPDWGVALGWCMIVFCIIWIPIMAIIKIIQAKG-NIFQRLISCCRPASNWGPYLEQHRGERYKDMVDPK--------
                                   P554L M560V

                   V629I
A8   621 ---------VSESSKVVVVESVM-------
RN   621 ---------VSESSKVVVVESVM-------
MM   626 ---------VSESSKVVVVESVM-------
BT   621 ---------VSESSKVVVVESVM-------
OC   621 ---------VSESSKVVVVESVM-------
TM   597 ---------VSCEEKVVIFESVL-------
A1   586 ---------AQAG-SSTSKEAYI-------
A2   607 ---------QFQLQHWLAI-----------
A3   610 ---------QFTLRHWLKV-----------
A4   626 ---------LNAV-----------------
A5   779 ---------SSLGLKLPVKDLELGTQC---
A6   599 ---------RTVMNGALVKPTHIIVETMM-
A7   607 HMRKYGGITSFENTAIEVDREIAEEEESMM
A9   613 QSLHP----DKAQIPIVGSNGSSRLQDSRI
A11  618 ---------DGTIAAITEKETHF-------
A12  600 ---------TREGLIAGEKETHL-------
A13  588 ---------RTSLLRLTELESHC-------
A14  625 ---------KEADHEIPTVSGSRKPE----
```

materials (e.g., fibroblasts or urine) were available, the pathogenicity could not be studied at the biochemical level or by H-MRS of the brain.

In two affected males of family N87, the transversion C→G at *SLC6A8* c.1011 was found, resulting in the substitution p.C337W. C337 is located in the intracellular loop between TM VI and VII and is highly conserved (fig. 2). The substitution of cysteine by tryptophan is highly significant: cysteine is polar and weakly acidic, and the sulfhydryl group is often involved in protein folding by forming disulfide links, whereas tryptophan is a nonpolar and neutral amino acid, with a very rigid cyclic structure. The patients' sister was a carrier of the p.C337W mutation (fig. 3*B*). In urine of the two affected males, an

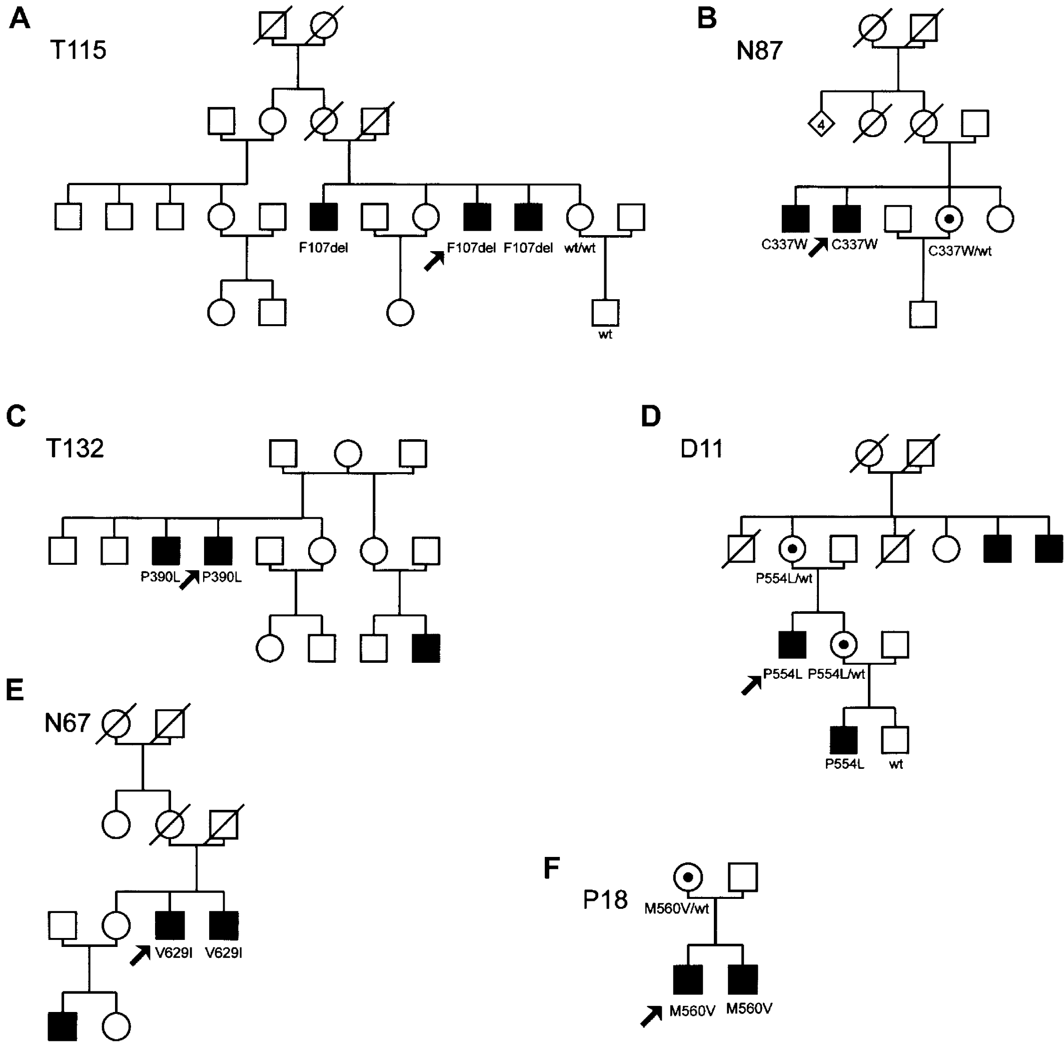

Figure 3 Pedigree charts of families in which segregation could be studied. (For designation of the variants, see table 1.)

increased Cr:Crn ratio was found, a biochemical hallmark of SLC6A8 deficiency. These data indicate that the p.C337W is pathogenic.

In DNA of the index patient of family T132, a transition of c.1169C→T was detected, resulting in the substitution p.P390L. The residue P390 is located in the extracellular loop between TM VII and VIII and is conserved in all known SLC6 family members, except for the serotonine transporter (fig. 2). The p.P390L substitution may have a significant effect on correct protein folding. An affected brother of the index patient also had the p.P390L substitution (fig. 3C). Therefore, we consider p.P390L a pathogenic mutation. It is unfortunate that, because of the unavailability of fibroblasts or urine, the pathogenicity could not be studied at the biochemical level or by H-MRS of the brain.

In DNA of the index patient of family D11, a transition of c.1661C→T was detected in exon 12 of *SLC6A8*, which resulted in the substitution p.P554L. P554 is a residue of the extracellular loop between TM XI and XII. The region

of TM IX–XI is expected to harbor the determinants of substrate specificity. The residue p.P554 is highly conserved (see fig. 2). The effect of a substitution of proline by leucine was discussed above. The p.P554L mutation segregates with the clinical phenotype within the family (fig. 3*D*). Thus, p.P554L is pathogenic. As discussed above, because of the lack of appropriate biological material from the affected males, the pathogenicity could not be studied at the biochemical or clinical level.

In DNA of the index patient of family N67, a G→A transition was detected at *SLC6A8* c.1885, which resulted in the substitution p.V629I in exon 13. The nonsynonymous substitution is located in a short repeat of four valines in the intracellular C-terminus of the creatine transporter protein (fig. 2). This residue is conserved in the creatine transporters between species but not among the SLC6 family members. Moreover, both valine and isoleucine are members of the same group of aliphatic amino acids, and, therefore, no change of chemical properties is predicted. The p.V629I mutation was also present in the affected brother (fig. 3*E*). Cr:Crn ratios in urine were within the low normal range (index patient and brother 0.001; control values 0–0.3). It is not known whether mutations in specific SLC6A8 regions could alter the function, stability, or trafficking of the protein, as is described for SLC6A2 (Bauman and Blakely 2002) and SLC6A13 (Brown et al. 2003). The c.1885G→A transition was not found in 276 controls and 95 ESTs. p.V629I is currently considered to be an unclassified variant.

In DNA of the index patient of family P18, an A→G transition was found in *SLC6A8* at c.1678 in exon 12, resulting in the substitution p.M560V. The substitution was also present in the affected brother of the index patient. The mother is a carrier of the transition (fig. 3*F*). p.M560 is conserved in rat, rabbit, and mouse creatine transporter but not in bovine (Val), marbled electric ray (Ile), or other members of the SLC6 family (fig. 2). The p.M560V mutation was not found in 276 control chromosomes. However, fibroblasts of the patient are not deficient in creatine uptake, and, in the H-MRS of the brain, the creatine signal was present. Therefore, we conclude that p.M560V is a rare polymorphism. Whether any of the above-mentioned silent, IVS, and 3′ UTR variants (table 1) are disease causing or just represent rare polymorphisms remains to be investigated.

The absence of missense mutations in 276 control chromosomes (appendix A) reduces the chance to <1% for a missense mutation to be a polymorphism with 80% power (Collins and Schwartz 2002). The size of the control population is rather small to provide conclusive information on the nature of a specific missense mutation found in the equal-sized XLMR population. On the other hand, the number of missense mutations differs significantly between the patient group and control groups (6/288 versus 0/276; Fisher's exact test *P* value = .03).

We have identified 6 pathogenic mutations in 288 patients. This means a prevalence of 2.1% SLC6A8-deficient patients in this XLMR panel. This may be an underestimate, since we were able to investigate only 93% of the coding sequence in 288 patients (the complete coding sequence in 180 patients and, overall, 80% of the coding sequence in the remaining 108 patients). In addition, some of the unclassified variants ($n = 11$) may prove to be pathogenic.

The presented results were calculated on the basis of our findings in a group of 288 patients who were preselected for suspected X-linked inheritance of MR. Currently, collaboration is being initiated to study the contribution of SLC6A8 deficiency in males with MR but without molecular defects in the *FMR1* gene (i.e., fraX negative).

Throughout exons 1–13 of the coding region of the *SLC6A8* gene, 14 previously reported and novel sequence variants have been identified. Mutation types include three nonsense mutations, two single–amino acid deletions in four families, one large deletion, four missense mutations, one combined missense/splice-site mutation, one unclassified variant (missense type), one translational silent mutation with unknown consequence, and one missense-type polymorphism. Although several mutations have been identified already, there is no indication of a recurrent mutation or a mutation-sensitive/-prone site (fig. 1).

Knockout-mouse models and clinical trials may result in a better understanding of the disorder and in appropriate treatment protocols for both affected males and females. The authors suggest that male patients with MR, autistic behavior, epilepsy, and/or expressive speech and language delay should be tested for creatine-deficiency disorders. Screening could be based on metabolite measurement of urine, H-MRS of the brain, and/or mutational analysis by direct sequencing of the gene(s). Functional tests could prove the biochemical defect and should include tests for AGAT and GAMT enzyme activity or creatine uptake into lymphoblasts or fibroblasts, respectively. SLC6A8 deficiency may contribute, together with fraX, to MR in an extensive proportion of males with nonsyndromic XLMR of unknown cause.

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Appendix A

Polymorphisms in the *SLC6A8* Gene

The anonymous DNAs of unaffected males were kindly provided by the Departments of Human Genetics of the VU University Medical Center in Amsterdam, the University Medical Center in Nijmegen, and the University Medical Center in Leiden.

SLC6A8 Polymorphisms in Controls and Patients with XLMR

IVS1+26G→A
IVS7+37G→A
IVS7+87A→G
IVS7-35G→A
IVS12-3C→T
c.1494C→T: p.Y498

SLC6A8 Polymorphisms in Controls Only

IVS2+88G→C
IVS9-36G→A
IVS12-82G→C

Electronic-Database Information

The accession number and URLs for data presented herein are as follows:

BLAST, http://www.ncbi.nlm.nih.gov/BLAST/
European XLMR Consortium, http://www.euromrx.com/
GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *SLC6A8* [accession number Z66539])
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *SLC6A8,* AGAT deficiency, GAMT deficiency, and SLC6A8 deficiency)

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Chapter 4

X-linked creatine transporter (SLC6A8) mutations in about 1% of males with mental retardation of unknown etiology.

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ORIGINAL INVESTIGATION

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X-linked *creatine transporter* (*SLC6A8*) mutations in about 1% of males with mental retardation of unknown etiology

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Abstract Mutations in the creatine transporter gene, *SLC6A8* (MIM 30036), located in Xq28, have been found in families with X-linked mental retardation (XLMR) as well as in males with idiopathic mental retardation (MR). In order to estimate the frequency of such mutations in the MR population, a screening of 478 males with MR of unknown cause was undertaken. All 13 exons of *SLC6A8* were sequenced using genomic DNA. Six novel potentially pathogenic mutations were identified that were not encountered in at least 588 male control chromosomes: two deletions (p.Asn336del, p.Ile347del) and a splice site alteration (c.1016+2C>T) are considered pathogenic based on the nature of the variant. A mutation (p.Arg391Trp) should be considered pathogenic owing to its localization in a highly conserved region. Two other missense variants (p.Lys4Arg, p.Gly26Arg) are not conserved but were not observed in over 300 male control chromosomes. Their pathogenicity is uncertain. A missense variant (p.Val182Met), was classified as a polymorphism based on a normal creatine/creatinine (Cr:Crn) ratio and cerebral creatine signal in proton magnetic resonance spectroscopy (H-MRS) in the patient. Furthermore, we found 14 novel intronic and neutral variants that were not encountered in at least 280 male control chromosomes and should be considered as unclassified variants. Our findings of a minimum of four pathogenic mutations and two potentially pathogenic mutations indicate that about 1% of males with MR of unknown etiology might have a *SLC6A8* mutation. Thus, DNA sequence analysis and/or a Cr:Crn urine screen is warranted in any male with MR of unknown cause.

Keywords Creatine transporter · X-linked mental retardation · Non-fragile X MR

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Introduction

X-linked mental retardation (XLMR) comprises a group of heterogeneous conditions with an estimated frequency of 5–12% in the mentally retarded population (Stevenson et al. 2000). It is likely that over 150 genes are associated with XLMR (Stevenson and Schwartz 2002). This would mean that mutations in a single gene would account for a small percentage of males with mental retardation (MR). Indeed, frequency data published to date for known XLMR genes are in agreement with this perception (Mandel and Chelly 2004). Fragile X is the most common cause of MR in males with a frequency of ~2.5% in the male MR population, and many other genes, especially those associated with nonsyndromic XLMR have a frequency of about 1/300–1/1,000 in the male MR population (Mandel and Chelly 2004). A possible exception is the *SLC6A8* (*creatine transporter*) gene located in Xq28. The *SLC6A8* gene encodes for a protein that transports creatine into cells. The first observed mutation in this gene was identified in a boy with MR who had severe speech and language delay, cerebral creatine deficiency and increased urinary creatine/creatinine ratio (Cecil et al. 2001; Salomons et al. 2001). Subsequently, a mutation was found in a family with XLMR which clearly demonstrated *SLC6A8* as an XLMR gene (Hahn et al. 2002). To date, 16 families with *SLC6A8* mutations have been reported including two families where the mutation arose *de novo* in the proband (Almeida et al.

2004; Bizzi et al. 2002; Cecil et al. 2001; Degrauw et al. 2002; Degrauw et al. 2003; Hahn et al. 2002; Mancini et al. 2005; Rosenberg et al. 2004; Salomons et al. 2001, 2003; Schiaffino et al. 2005) This number includes the six families that recently were identified in an analysis of 288 males with presumed XLMR based on pedigree information, leading to a pathogenic mutation prevalence of about 2% (Rosenberg et al. 2004).

In order to estimate the frequency of mutations in *SLC6A8* in a more general MR population, we conducted a molecular-based screening of 478 males with non-fragile X MR of unknown etiology. We identified a minimum of four pathogenic mutations, which gives a frequency of at least 0.8% (CI 0.02–1.7%). Thus, mutations in the *creatine transporter* gene, *SLC6A8*, may be a relatively major contributor compared to most other XLMR genes with the exception of *FMR1* and *ARX* (Mandel and Chelly 2004; Stepp et al. 2005) to MR in the male population.

Methods

Patient information

A group of 478 unrelated male patients with MR of unknown cause, all of whom receive services through the South Carolina Department of Disabilities and Special Needs (DDSN), were screened for mutations in the *SLC6A8* gene. This group comprised of 254 blacks, 211 whites, three from other backgrounds and ten individuals with unknown racial or ethnic status. Four hundred and fifty seven patients had IQ scores available. Of these, 89 (19.5%) had profound MR, 87 (19%) had severe MR, 103 (22.5%) had moderate MR, 165 (36.1%) had mild MR and 13 (2.8%) had IQs in the normal range, but deficits in adaptive behavior. All patients had undergone a clinical genetics/dysmorphology examination and 97% had been karyotyped. In addition, 86% of patients had previously undergone molecular testing for the trinucleotide repeat expansion in the *FMR1* gene and were found to be negative. An additional 7% were negative by cytogenetic testing for Fragile X.

Sequencing

All 13 exons of *SLC6A8* were sequenced using genomic DNA as described previously (Rosenberg et al. 2004) using BigDye v3.1 terminators and an ABI3100 machine (Applied Biosystems, Nieuwekerk a/d Ijssel, The Netherlands). The obtained sequences were analyzed using Mutation Surveyor software package (Softgenetics, State College, PA, USA).

Polymorphism analysis

The four missense variants and the splice site alteration were analyzed in a cohort of at least 305 normal male chromosomes (58% Caucasian, 12% African American, 2% Hispanic, 28% unknown) using enzymatic digestion of PCR products to determine the presence or absence of the base change (Table 1). Additionally, 280 normal control male chromosomes (Caucasian) were sequenced for the appropriate exons using genomic DNA as previously described (Rosenberg et al. 2004).

Results

Sequencing of genomic DNA from a cohort of 478 males with MR of unknown etiology identified six potential pathogenic mutations, four novel translational silent alterations, and one missense polymorphism within the *SLC6A8* gene (table). Of the six potential pathogenic mutations, two were small deletions, one was a splice site alteration and three were missense variations/mutations (Fig. 1). Furthermore, nine novel intronic variants and five neutral variants were encountered that were not seen in a minimum of 280 male control chromosomes (Rosenberg et al. 2004). In addition, one known and one novel silent change were found in the MR cohort as well as in male control chromosomes and the p.Met560Val was found only in a control (present control cohort of four males) and thus these are considered polymorphisms (Table 1).

In genomic DNA of patient CMS5804, a deletion of three base pairs (c.1006_1008delAAC) was detected in exon 6 of the *SLC6A8* gene, which resulted in the loss of asparagine at protein position 336 (p.Asn336del). The deletion is located in a short repeat of two asparagines in a small intracellular loop between transmembrane (TM) domains VI and VII, which is highly conserved among all known creatine transporters (i.e. bovine, rat, rabbit, marbled electric ray and mouse) and within the human Na^+ and Cl^- dependent neurotransmitter transporter family (multiple sequence alignment: see ref. Rosenberg et al. 2004 Fig. 2). Taken together, p.Asn336del is considered pathogenic based on the fact that p.Asn336 is highly conserved between species, resides in a highly conserved region within its solute carrier family, and was not found in 280 male control DNAs. The 6-year-old patient has moderate MR (IQ = 46), ADHD and seizures, is tall and thin, and has an OFC of 47.5 cm (< 2 percentile). It is of note that a maternal nephew has MR. Unfortunately, we were unable to re-examine this individual or other members of his family.

In genomic DNA from patient CMS4963, a deletion of three base pairs (c.1040_1042delTCA) was detected in *SLC6A8* exon 7, which results in the loss of isoleucine at position p.347 (p.Ile347del). The mutation is located in the highly conserved TM domain VII. Biochemically, an increased urinary Cr:Crn ratio (2.1; normal range 0.01–0.53) was observed. The nature of the mutation, its conserved location, the increased urinary Cr:Crn ratio, and the absence of the deletion in 280 male controls indicate that p.Ile347del is a pathogenic mutation. Clinical findings in this 26-year-old patient are moderate

Table 1 Pathogenic mutations and DNA alterations in *SLC6A8* detected in a cohort of 478 male MR patients and/or a minimum of 280 controls

Patient	c.DNA	Deduced effect	Exon	Controls			Cr : Crn[b]	IQ	Seizures	Other features
				RFLP		Rosenberg et al. 2004[a]				
Pathogenic mutations										
CMS5804	c.1006_1008delAAC	p.Asn336del	6	ND		0/280	NA	46	Y	ADHD; microcephalic; tall and thin; FHMR
CMS4963	c.1040_1042delTCA	p.Ile347del	7	ND		0/280	2.1	36	Y	Aggressive behavior; tall, thin; wide-spaced eyes; no FHMR
CMS5183	c.1016 + 2C > T	Splice error		Cac B1	0/356	0/280	1.4	11	Y	Short stature; HC ~10th percentile; large testes
CMS5186	c.1171C > T	p.Arg391Trp	8	Sac II	0/356	0/280	NA	35	Y	Short stature; unknown family history
Potentially pathogenic missense mutations										
CMS4983	c.11A > G	p.Lys4Arg	1	Sac I	0/348	0/280	NA	63	N	Short stature; long, narrow face; flat midface; prominent chin; no FHMR
CMS5058	c.76G > A	p.Gly26Arg	1	Ava I	0/375	0/280	NA	24	N	Long thin face; flat midface; prominent chin; no FHMR
Polymorphisms										
CMS5567	c.544G > A	p.Val182Met	3	Hpy Ch4V	1/355	0/280	0.02	< 20	Y	[c]
CMS5178	c.813C > T		5	Ava II	0/337	4/280				
CMS5167	c.1494C > T		10	Rsa I	0/305	1/280				
Control male	c.1678A > G	p.Met560Val	12			1/280				
Neutral variants that were not found in controls										
CMS5771	c.603C > T		3	ND		0/280				
CMS5130	c.780C > T		5	ND		0/280				
CMS5122[d]	c.1437C > T		10	ND		0/280				
CMS5800	c.1662G > A		12	ND		0/280				
Intronic variants that were not found in controls										
CMS4946[e]	c.262 + 53G > C			ND		0/280				
CMS4922[f]	c.1016 + 41_45dupTGCCC			ND		0/280				
CMS5140[g]	c.1017-38C > G			ND		0/280				
CMS4992[h]	c.1142-130C > T			ND		0/280				
CMS5530	c.1254 + 39G > T			ND		0/280				
CMS5511	c.1255-59C > T			ND		0/280				
CMS5810	c.1392 + 31T > C			ND		0/280				
CMS5008	c.1496-5C > T			ND		0/280				

ND not performed, *N* no, *Y* yes, *NA* no material available, *FHMR* Family history of MR, *NM* not measured

[a] The coding region of the SLC6A8 gene was analyzed by DNA sequence analysis of 276 male control chromosomes (Rosenberg et al 2004) plus that of four male control chromosomes (present study).

[b] *Cr:Crn* creatine/creatinine ratio, *normal range* 0.01–0.053

[c] The variant did not segregate with the phenotype in the family and the patient had a normal creatine signal in H-MRS of brain.

[d] Alteration found in 3 other unrelated individuals: CMS5496, CMS5510, CMS7508

[e] Alteration found in 2 other unrelated individuals: CMS5041, CMS5130

[f] Alteration found in 10 other unrelated individuals: CMS5033, CMS5046, CMS5056, CMS5181, CMS5491, CMS5820, CMS5490, CMS5491, CMS5492, CMS5527

[g] Alteration found in 4 other unrelated individuals: CMS5147, CMS5165, CMS5843, CMS5489

[h] Alteration found in 12 other unrelated individuals: CMS4994, CMS5034, CMS5041, CMS5042, CMS5046, CMS5130, CMS5491, CMS5779, CMS5820, CMS5490, CMS5491, CMS5492

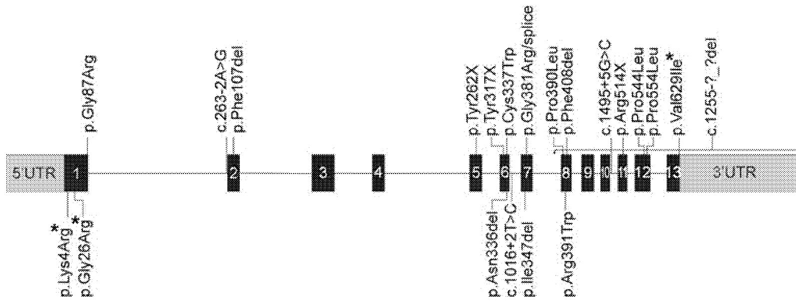

Fig. 1 Mutations and unclassified variants in the *SLC6A8* gene. Sequence variants were annotated according to the guidelines of den Dunnen and Antonarakis (http://www.hgvs.org/mutnomen) and presented by their deduced effect: p.Lys4Arg (c.11A > G), p.Gly26Arg (c.76G > A), p.Asn336del (c.1006_1008delAAC), c.1016 + 2T > C, p.Ile347del (c.1059_1061delCTT), p.Arg391Trp (c.1171C > T). The mutations/unclassified variants identified in the present study are depicted below the schematic of the gene. References for previously published mutations/variations are: p.Gly87Arg (c.259G > A), p.Phe107del (c.319_321delCTT), p.Tyr317X (c.950_951insA), p.Cys337Trp (c.1011C > G), p.Pro390Leu (c.1169C > T), p.Pro554Leu (c.1661C > T) and p.Val629Ile (c.1885G > A): Rosenberg et al. 2004; c.263-2A > G: Schiaffino et al. 2005; p.Phe107del (c.319_321delCTT), p.Tyr262X (c.786C > G), p.Phe408del (c.1221_1223delCTT), p.Gly381Arg/splice (c.1141G > C), p.Arg514X (c.1540C > T), c.1255?_?del: Salomons et al. 2003; p.Gly381Arg/splice (c.1141G > C): Hahn et al. 2002; p.Phe408del (c.1221_1223delCTT): Bizzi 2002, deGrauw et al. 2002, Salomons et al. 2003; c.1495 + 5G > C, p.Pro544Leu (c.1631C > T): Mancini et al. 2004; p.Arg514X (c.1540C > T): Salomons et al. 2001, 2003, deGrauw et al. 2002; p.Phe107del (c.319_321delCTT), p.Tyr262X (c.786C > G), p.Phe408del (c.1221_1223delCTT), p.Arg514X (c.1540C > T): deGrauw et al. 2002. * indicates that these DNA changes are unclassified variants

MR (IQ = 36), aggressive behavior, seizures, a tall and thin posture and long fingers. There is no history of MR in this family. The family was not interested in follow-up studies.

Patient CMS5183 was found to have a T > C transition in intron 6 (c.1016 + 2T > C). The mutation affects the classical splice donor dinucleotide motive (GT) and is predicted to result in aberrant splicing of the mRNA. Analysis using five web-based splice predictor tools (Fruitfly, Genscan, NetGene2, SplicePredictor, SpliceSiteFinder) showed without exception, that the splice donor recognition site is abolished. The patient was unavailable for further study of either lymphocytes or fibroblasts to prove incorrect splicing. However, urine had been stored and an elevated Cr:Crn ratio (1.4; normal range 0.01–0.53) was detected. These findings and the fact that this alteration was not observed in 280 controls nor in 356 normal X chromosomes examined using a restriction endonuclease digestion (table), indicate that the c.1016 + 2T > C mutation is pathogenic. Clinical features of this deceased patient were severe MR (IQ = 11), seizures, short stature, small head circumference (54 cm, 10th percentile), and large testicles. Family history was unknown for MR.

A C > T transition at position c.1171 in exon 8 of the *SLC6A8* gene was observed in patient CMS5186. This transition results in the substitution p.Arg391Trp. The residue p.Arg391 is located in the extracellular loop between TM domains VII and VIII. This extracellular loop is highly conserved between species and within the SLC6 family. At position p.391, only the electric ray carries another positively charged amino acid, Lys. Within the SLC6 family, p.Arg391 itself is not conserved but tryptophan never occurs at this position. Arginine is positively charged and highly hydrophilic, whereas tryptophan is aromatic and highly hydrophobic. The opposite chemical properties of the wild type and mutant amino acid, the location in a highly conserved region, and the fact that c.1171C > T was not found in 280 control chromosomes, nor in 356 control male X chromosomes examined using a restriction endonuclease digestion (table), indicate that the p.Arg391Trp missense mutation is very likely pathogenic. Clinical findings in the 41-year-old patient are moderate MR (IQ = 35), seizures and short stature. Because no additional biological materials, e.g. fibroblasts or urine were available, the pathogenicity could not be studied at the biochemical level. Additionally, it was not possible to obtain MRS imaging of the patient's brain.

Genomic DNA of patient CMS5058 was found to have a G > A transition at position c.76 in exon 1 of *SLC6A8*, which results in the substitution p.Gly26Arg. Between species, glycine at position 26 is conserved in SLC6A8 except for the electric ray where glycine is substituted with a proline. Both glycine and proline are aliphatic neutral amino acids. Arginine, however, is a positively charged and highly hydrophilic amino acid, which differs from the biochemical properties of glycine and proline. No additional biological material on the patient was available. The p.Gly26Arg missense change is considered an unclassified variant. Clinical findings in this 44-year-old patient are severe MR (IQ = 24), long thin face, flat mid face with a prominent chin and no family history of MR.

In genomic DNA of patient CMS4983, the transition of A > G at position c.11 in exon 1 of the *SLC6A8* gene was found. This transition results in the replacement of lysine at residue 4 by arginine (p.Lys4Arg). The p.Lys4Arg substitution occurs in a region that is conserved in the creatine transporter between species (mouse, rat,

bovine and rabbit), but in electric ray, the fourth position is indeed occupied by arginine. Both lysine and arginine are positively charged, basic, highly hydrophilic amino acids. Furthermore, the *N*-terminus of the transporter is not conserved within the solute carrier family. However, the substitution was not encountered in 624 controls (table) and is, therefore, an unclassified variant. Clinical findings in this 15-year-old patient are mild MR (IQ = 63), short stature and a long narrow face with a prominent chin. Additional follow-up has not been possible.

In patient CMS5567, a G > A transition was found at position c.544 in exon 3 of *SLC6A8*. This results in the substitution p.Val182Met. The Val182 residue is located in the extracellular loop between TM domain III and TM domain IV. This region is not conserved between species, or within the superfamily of neurotransmitter transporters. Although, the p.Val182Met change substitutes one hydrophobic residue with another, it was not found in 655 normal X chromosomes (table). However, H-MRS of the brain of patient CMS5567 revealed a normal creatine signal, and urinary Cr:Crn was within the normal range (table). Additionally, analysis of multiple affected brothers of patient CMS 5567 revealed a lack of segregation of the change with the MR phenotype. Therefore, p.Val182Met is considered a rare polymorphism.

In the MR panel, four variants in patients (c.603C > T, c.780C > T, c.1437C > T, c.1662G > A) were encountered that were not found in 280 male control chromosomes (table). These four transitions are neutral as they do not result in amino acid substitutions. Bioinformatic analysis did not indicate any of these transitions would adversely affect splicing. However, no further material was available for further workup of these patients. Therefore, we consider these substitutions as unclassified variants.

Additionally, two variants, c.813C > T and c.1494C > T, were found both in patients as well as in our control cohort (table). Therefore, these variants are considered to be polymorphisms.

Discussion

X-linked mental retardation (XLMR) is estimated to account for 5–12% of the MR population (Stevenson et al. 2000). However, XLMR is comprised of a heterogeneous group of conditions, so, it is very difficult for a clinician to know with certainty if any given male with MR has XLMR. Moreover, the X-linked mode of inheritance will not be observed in the case of a de novo mutation. Furthermore, it is estimated that over 150 genes are associated with XLMR (Stevenson and Schwartz 2002). The combination of these factors means a combined total of mutations in any single XLMR gene will have a frequency of roughly 1/300–1/1,000 (Mandel and Chelly 2004). Thus, the identification of an XLMR gene with a frequency approaching 1% in the male MR population would be a significant finding. One such potential gene is the *SLC6A8* gene, located in Xq28.

We conducted a screening of 478 unrelated males with MR for mutations in the *SLC6A8* gene. This cohort was not preselected for severity of MR or family history of MR. The DNA samples of these MR patients were randomly selected. Patients were excluded if they had a positive fragile X test or if another recognizable phenotype was previously diagnosed. In total, 86% of the cases tested in the present study were negative for fragile X, by molecular testing and an additional 7% were negative by cytogenetic testing. Thus, our analysis should allow an estimation of the frequency of *SLC6A8* mutations in a broad population of males with MR of unknown etiology.

In this cohort we found four novel pathogenic and two novel potentially pathogenic mutations (see figure: lower panel). Two out of the four pathogenic mutations were single amino acid deletions (p.Asn336del, p.Ile347del). This type of mutation was also reported in 3 out of 16 previously reported SLC6A8 mutations (Almeida et al. 2004; Bizzi et al. 2002;Cecil et al. 2001; Degrauw et al. 2002; Degrauw et al. 2003; Hahn et al. 2002; Mancini et al. 2005; Rosenberg et al. 2004; Salomons et al. 2001, 2003; Schiaffino et al. 2005). The novel p.Asn336del and p.Ile347del mutations are directly next to or only 10 amino acids apart from the p.Cys337 residue that has been found to be mutated previously p.Cys337Trp, (Rosenberg et al. 2004) indicating the significance of amino acid conservation in this region. The third pathogenic mutation comprised a splice site error (c.1654 + 2C > T). Mutations that affect the splice donor or acceptor sites have been observed in two previously reported patients (c.263-2A > G and c.1495 + 5G > C). The fourth pathogenic mutation detected in the present cohort comprised a novel missense mutation (p.Arg391Trp). Six disease causing missense mutations have been reported previously in SLC6A8 (Almeida et al. 2004; Bizzi et al. 2002; Cecil et al. 2001; Degrauw et al. 2002, 2003; Hahn et al. 2002; Mancini et al. 2005; Rosenberg et al. 2004; Salomons et al. 2001, 2003; Schiaffino et al. 2005).

In total, 18 mutations (from 20 unrelated families) are reported, including the four mutations described in the present paper: three nonsense, seven missense, three classical splice mutations, four single amino acid deletions, and one large genomic deletion. The mutations are found throughout the gene. It is of note that so far no naturally occurring mutations in TM domain III have been described, the region that is shown to be involved in the substrate binding site (Dodd and Christie 2001, 2005). Furthermore, in the present study we identified two missense variants of unknown significance (p.Lys4Arg, p.Gly26Arg). Since these two unclassified variants are not located in conserved regions of the SCL6A8 species or the SLC6 family members these likely represent rare polymorphisms.

Our observation of four pathogenic mutations in 478 males gives a frequency of 0.8% (CI 0.02–1.7%), that

could rise to 1.3% (CI 0.26–2.3) if the two unclassified missense variants (p.Lys4Arg, p.Gy26Arg) turn out to be pathogenic. It should be noted that about 5% of the ORF of the 478 patients could not be scanned, due to poor quality of the amplicons/DNA. Also, the promotor region and the majority of the intronic sequences in all 478 samples were not analyzed. Moreover, 13 novel neutral or intronic variants were encountered that were not found in controls. Approximately half of the MR patients that receive services from the South Carolina DDSN have MR of unknown causation. Thus the "true" frequency in the overall male MR population is closer to 0.4–0.7% (half the number mentioned above).

The prevalence of 0.8% is lower than the 2.1% (CI 0.4–3.7%) observed in a previous study (Mandel 2004; Rosenberg et al. 2004). However, as the Rosenberg et al. (2004) study utilized males with a strong predilection to having XLMR, that observed frequency is expected to be higher than that expected in a cohort of male patients with MR of unknown cause. Conversely, if 10% of the present cohort of males with MR represents XLMR, the observed frequency of 0.8% (CI 0.02–1.7%) is higher than that expected (e.g. ~0.2%) based on the 2.1% (CI 0.4–3.7%) in the XLMR panel (Rosenberg et al. 2004). However, the relatively small sample size of both cohorts provides large CIs, which is in line with the observed findings. A bias towards XLMR in the present panel cannot be excluded. This is not likely, however, since in the five probands with a proven or potentially pathogenic mutation in the present paper have no history of MR in the family. The male with the p.Asn337del may have a nephew with MR of unknown cause according to medical records. On the other hand, the panel of the European XLMR consortium lacks males in whom a *de novo* mutation had arisen. *De novo* mutations may be a frequent event as this has been observed in two of the 16 (12.5%) previously reported patients. Nonetheless, the present data suggest that *SLC6A8* mutations will have a frequency about 3–5 times greater than most other XLMR genes (Mandel and Chelly 2004), and thus may be a major contributor to MR in the male population. Since the description of the first patient in 2001, 20 families, including the four in the present paper, have been published. Given that mutations in *SLC6A8* are associated with an increased creatine/creatinine ratio in urine, a urine screen in the male MR population should prove valuable and lead to a focused mutation testing at the DNA level in males with an increase in this ratio. Also, it needs to be noted that the urine specimen for patient CMS5183 was over 10 years old. This should allay concerns about the use of stored samples for these analyses. Moreover, this would allow detection of the biosynthesis defects (Almeida et al. 2004) other than SLC6A8 mutations that respond well to treatment that is based on creatine supplementation (Stockler-Ipsiroglu and Salomons 2006; Stromberger et al. 2003). The finding of a pathogenic mutation in *SLC6A8*, in turn, should provide valuable diagnostic information for many families with a male or males with previously unexplained MR.

Electronic-database information

The accession number and URLs for data presented herein are as follows:

Splice predictor programmes:

Fruitfly—http://www.fruitfly.org/seq_tools/splice.html

Genscan—http://genes.mit.edu/GENSCAN.html

NetGene2—http://cbs.dtu.dk/services/NetGene2/

SplicePredictor—http://bioinformatics.iastate.edu/cgi-bin/sp.cgi

SpliceSiteFinder—http://genet.sickkids.on.ca/~ali/splicesitefinder.html

OMIM—http://www.ncbi.nlm.nih.gov/OMIM

SLC6A8 gene–Genomic–Accession Z66539, GI:1628386

mRNA–Accession NM_005629.1, GI:5032096

Mutation description—http://www.hgvs.org/mutnomen

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Chapter 5

Functional characterization of missense variants in *creatine transporter/SLC6A8*: improved diagnostic application.

submitted

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Functional characterization of missense variants in *creatine transporter/SLC6A8*: improved diagnostic application

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Abstract

Creatine transporter deficiency is an X-linked mental retardation disorder caused by mutations in the creatine transporter gene (*SLC6A8*). So far, 20 mutations in the *SLC6A8* gene have been described. We have developed a diagnostic assay to test creatine uptake in fibroblasts. Additionally, we expanded the assay to characterize novel *SLC6A8* missense variants. Thirteen variants were introduced in the *SLC6A8* cDNA by site directed mutagenesis. All variants were transiently transfected in SLC6A8 deficient fibroblasts and tested for restoration of creatine uptake in deficient primary fibroblasts. Thus, we proved that nine variants (p.Gly87Arg, p.Phe107del, p.Tyr317X, p.Asn336del, p.Cys337Trp, p.Ile347del, p.Pro390Leu, p.Arg391Trp, p.Pro554Leu) are pathogenic mutations and four variants (p.Lys4Arg, p.Gly26Arg, p.Met560Val, p.Val629Ile) are non-pathogenic. The present study provides an improved diagnostic tool to classify sequence variants of unknown significance.

Introduction

Deficiency of the creatine transporter gene (*SLC6A8/CRT/CT1*; [MIM 300036]) is an X-linked form of the cerebral creatine deficiency syndromes (CCDS). Common clinical features are mental retardation, expressive speech and language delay, autistic behaviour, and/or epilepsy. Creatine (Cr) and phosphocreatine play essential roles in the storage and

transmission of phosphate-bound energy [1,2]. Cellular creatine transport is of fundamental importance for Cr homeostasis in tissues void of robust creatine biosynthesis. The uptake of creatine is mediated by the creatine transporter protein. The *SLC6A8* gene (GeneID 6535) has been mapped to Xq28 [3]; it spans ~8.4 kb, consists of 13 exons and encodes a protein of 635 amino acids with a predicted molecular weight of 70 kDa [4]. Since the description of the first patient [5], 20 mutations have been described throughout the *SLC6A8* gene [5-13].

Previously, we have estimated the prevalence of SLC6A8 deficiency to be 2.1 % (CI: 0.44-3.8%) in an European X-linked mental retardation panel [7] (European Mental Retardation Consortium: http://www.euromrx.com/). Two additional studies reported prevalences of approximately 0.8 and 2% in patients with idiopathic mental retardation [12,14].

The sequence variants that were identified in the *SLC6A8* gene of mentally retarded patients include clear pathogenic DNA variants, such as single amino acid deletions, splice errors and nonsense mutations, but also missense mutations, which are more difficult to interpret. The latter poses problems for adequate molecular diagnosis. In the first prevalence study [7], six presumed pathogenic mutations were detected, and two unclassified missense variants. In the second study [12] six potentially pathogenic mutations were detected. Of these, four variants were considered pathogenic based on the absence of the variant in controls (n=280), conservation of SLC6A8 between species and the human functionally related proteins (SLC6), and segregation within the family.

In the present paper we describe an *in vitro* method for the diagnosis of SLC6A8 deficiency in fibroblasts using gas chromatography mass spectrometry and stable isotope labeled creatine as internal standard (SID-GCMS). The test is validated and applied to cell lines of 13 unaffected individuals and 12 affected patients. Difficulties in distinguishing disease-causing missense mutations from rare variants especially arise when no supplemental material (e.g. fibroblasts, proton magnetic resonance spectroscopy) is available to substantiate the clinical

diagnosis of SLC6A8 deficiency. Therefore, we expanded the creatine uptake assay to study the pathogenic nature of variants by *in vitro* introducing the mutation in an expression vector with the *SLC6A8* coding sequence, followed by transient transfection in SLC6A8 deficient fibroblasts.

MATERIALS AND METHODS

Fibroblasts cell culture

Thirteen fibroblast cell lines in which no inborn errors of metabolism were detected, were anonymized and used as control cell lines. Fibroblast cell lines of twelve patients, affected with creatine transporter deficiency, were reported previously [5,7,8,10]. Fibroblasts were cultured in culture flasks (Greiner Bio-One) in HAM-F10 supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (GIBCO) under a humidified atmosphere of 95% air/5% CO_2 at 37°C. Culture medium in the absence or presence of 10% FBS was analyzed for Cr content by GCMS (see below) and contained 0 and 25 µM creatine, respectively.

Creatine uptake assay

Creatine monohydrate (50 mM, Fluka) and β-guanidinopropionate (GPA) (100 mM, Sigma) stock solutions were dissolved in Hanks' Balanced Salt Solution (HBSS). Both stock solutions were filter sterilized (0.22 µm filter) and stored at –20°C. The optimal testing conditions were determined by incubation with a concentration range of 25-1000 µg/µl Cr. After 24 hours, the cells were pelleted and stored at -80°C until further use. When fibroblasts of SLC6A8 deficient patients were incubated with 500 µM up to 1000 µM creatine some intracellular Cr (6-17 pmol Cr/µg protein) could be detected.

Cells were incubated for 2 and 24 hours with 500 μM Cr, in presence of 100, 500 and 1000 μM β-guanidinopropionate (GPA), an inhibitor of SLC6A8 [15]. After a 2 hours incubation with 500 μM creatine and 500 μM GPA no significant decrease was found in creatine uptake of control fibroblasts, in comparison to the incubation with 500 μM creatine only. When the incubation time was extended to 24 hours, a twofold decrease in creatine uptake was found with 500 and 1000 μM GPA in control fibroblasts.

Twelve SLC6A8 deficient patient fibroblast cell lines and 13 control fibroblasts were tested with 25 μM Cr, 500 μM Cr and 500 μM Cr + 500 μM GPA, respectively. Cells were plated into 6 well culture plates (Greiner Bio-One) and cultured for at least 24 hours in order to obtain 50-80% confluent cell layers. Cells were incubate for 24 hours with or without 500 μM creatine in the presence or absence of 500 μM GPA. Cells were harvested by trypsinisation, pelleted (2 min. 4000 g) and stored at –80°C. before they were further processed for the quantification of creatine by gas chromatography mass spectrometry (GCMS), using stable isotope labeled creatine as internal standard.

Cell pellets were resuspended in 102 μl HBSS and 60 μl was used for analyzing intracellular creatine concentration. 2.5 nmoles of $^{2}H_3$-creatine was added as internal standard. Creatine is converted to a pentafluorobenzyl derivate which can be quantitatively measured by the SID-GCMS method as described before [16,17]. Protein content was determined using the Bicinchoninic acid protein assay kit (Sigma) according to the instructions of the manufacturer. The creatine concentration is expressed as pmol creatine/μg total protein.

Construction of *SLC6A8* expression vector

RNA was isolated from control fibroblasts (Promega SV RNA isolation kit) and cDNA was synthesised by reverse transcriptase (Qiagen). The full length SLC6A8 open reading frame (ORF) was amplified by PCR using specific primers, with *Bam*HI or *Hind*III restriction site

extensions. Forward primer 5'-CCC**AAGCTT**CCACCATGGCGAAGAAGAGCGCCGAG-3', reverse primer 5'-CGC**GGATCC**ATGACACTCTCCACCACGAC- 3'. This amplicon was cloned into pCR2.1-TOPO (Invitrogen). The construct (pCR2.1-*SLC6A8*) was used for subcloning into pEGFP-N1 or as template for site directed mutagenesis

Site Directed Mutagenesis

Site directed mutagenesis was used to introduce the mutations/variants in pCR2.1-*SLC6A8* using specific primers for each mutation/variant. The PCR reaction mix consisted of 50 ng pCR2.1-*SLC6A8*, 100 ng forward and reverse primer (sequences available upon request), 0,8 U Phusion polymerase (Bioké, Leiden, The Netherlands), 10 μg dNTP and 1x PCR buffer in a total volume of 50 μl. PCR reaction was performed with 50 cycles of 98°C for 10 seconds, 56-68°C (depending on the primers) for 30 seconds and 72°C for 90 secondes. After amplification, the template pCR2.1- *SLC6A8* was digested by incubation with DpnI at 37°C for two hours. Subsequently, the mutant *SLC6A8*-ORF was excised with BamHI and HindIII, and ligated into a pre-digested pEGFP-N1 expression vector (Clontech).

After cloning and subcloning, the SLC6A8 cDNA of all the different constructs was completely sequenced to confirm the presence of the desired mutation, and absence of PCR artefacts. Concentration and purity of the constructs was determined with the Nanodrop N-1000 according to the manufacturers directions. The A_{260}/A_{280} ratio of all constructs that were used for transfection was between 1.8 and 2.0.

Transfection

The SLC6A8 deficient primary fibroblasts (hemizygous for the p.Arg514X mutation) were used for transfection [5]. Cells were grown to ~70% confluence. The pEGFP-SLC6A8 constructs were incubated at room temperature for 10 minutes with polyethyleneimine (PEI,

Polysciences) in a 1:3 ratio in serum free medium and subsequently applied to the cells. The medium was refreshed after 4 hours. Incubation with creatine was started 48 hours after transfection. Transfections and the creatine uptake assay of each variant was performed in triplo (500 µM Cr), and measured by SID-GCMS. The creatine concentration results were corrected for the protein content.

RT-PCR

In order to prove expression of the constructs, RT-PCRs were performed. RNA isolation and cDNA synthesis from the transfected cells was performed as described above. A SLC6A8 specific forward primer (5'-CGTTGTGTACTACGAGCCG- 3') and EGFP specific reverse primer (5'-CGTCGCCGTCCAGCTCGACCAG- 3') were used to PCR amplicons of 376 bp. To rule out genomic and plasmid DNA contamination, RT-PCR was performed without Reverse Transcriptase for each transfected cell line.

RESULTS

Creatine uptake assay

Cr uptake was measured in control fibroblasts (n=13) and SLC6A8 deficient fibroblasts (n=12) The reference values obtained for both groups are listed in Table 1. In all 12 patient fibroblast cell lines with a pathogenic mutation in the *SLC6A8* gene, a significant difference in the creatine uptake profile was found compared to the 13 control cell lines. When SLC6A8 deficient fibroblasts were grown in the presence of a physiological Cr concentration (25 μM), the average creatine uptake (0.58 ± 1.03 pmol Cr/μg protein) was just above the detection limit, whereas in the control fibroblasts it was significantly higher (27.8 ± 5.6 pmol Cr/μg protein). In the presence of 500 μM Cr, SLC6A8 deficient fibroblasts showed an average creatine uptake of 6.22 ± 2.14 pmol Cr/μg protein, which is approximately 7 times lower than that in control fibroblasts (41.3 ± 7.0 pmol Cr/μg protein). SLC6A8 deficient fibroblasts incubated in the presence of 500 μM creatine and 500 μM GPA, showed a slight decrease of creatine uptake (6.22 to 4.58 pmol Cr/μg protein), whereas in control fibroblasts more than a twofold decrease was noted (41.3 to 17.7 pmol Cr/μg protein). The intra-assay and inter-assay value was shown to be 27.1 ± 1.2 (4%) and 27.8 ± 3.2 pmol Cr/μg protein (12%), respectively.

Transient transfection of pEGFP-SLC6A8 variants

Previously, we reported nine likely pathogenic variants, three unclassified variants and one non-pathogenic variant in the ORF of SLC6A8 (Table 2) [7,12]. To obtain final prove for the nature of these variants each of these variants was introduced in the *SLC6A8* ORF by site directed mutagenesis, subcloned in the pEGFP-N1 vector (pEGFP-Lys4Arg ... pEGPF-Val628Ile), expressed in SLC6A8 deficient primary fibroblasts, and tested for Cr uptake capacity when incubated with 500 μM Cr, as described above. RT-PCR confirmed mRNA

expression, indicating succesfull transfection. In contrast to the mock and non-transfected cells, expression of SLC6A8-EGFP mRNA was observed in all cells transfected with wild-type or variant pEGFP-SLC6A8 constructs.

There was no significant difference in creatine uptake between the non-transfected cells (6.2 pmol Cr/μg protein) versus mock transfectants (pEGFP-N1; 5.1 pmol Cr/μg protein; Figure 1) in the same assay. Transient transfection with wild-type pEGFP-SLC6A8 resulted in a 6-fold increase of Cr uptake (29.3 pmol Cr/μg protein; Figure 1) compared to the mock transfectants (5.1 pmol Cr/μg protein). Overexpression of nine variants (p.Gly87Arg, p.Phe107del, p.Tyr317X, p.Asn336del, p.Cys337Trp, p.Ile347del, p.Pro390Leu, p.Arg391Trp and p.Pro554Leu) did not result in a significant increase of Cr uptake (range 4.9 – 8.6 pmol Cr/μg protein: Figure 1). Overexpression of pEGFP-Lys4Arg, pEGFP-Gly26Arg, pEGFP-Met560Val and pEGFP-Val629Ile (23.5 - 34.3 pmol Cr/μg protein) resulted in a Cr uptake profile similar to the wild-type pEGFP-SLC6A8 (29.3 pmol Cr/μg protein Figure 1).

Discussion

The broad spectrum of sequence variants in the *SLC6A8* gene in (XL)MR patients poses problems with regard to classification of these changes as pathogenic mutations or non-pathogenic variants/polymorphisms. Deletions in *SLC6A8*, ranging from single nucleotides to contiguous gene deletions [18] or splice errors, and (other) truncating mutations can easily be classified as pathogenic mutations. However, in order to gain insight in the significance of missense variants, additional workup is needed. This is particularly true for the missense variants that we have identified in the patient panels studied for the prevalence of *SLC6A8* mutations [7,12]. In most cases it was impossible to prove the clinical diagnosis of SLC6A8 deficiency, since further investigations, such as urinary Cr/Crn ratio assessment and/or brain H-MRS imaging could not be performed. We now have developed and validated a functional diagnostic assay, in which we can determine Cr uptake activity in cultured primary fibroblasts, which is reliable and reproducible.

Previously, we have shown that SLC6A8 deficient primary fibroblasts can be complemented by stable transfection with the wild-type ORF of *SLC6A8* [19]. Since fibroblasts are difficult to transfect stably and are slowly proliferating, we have adapted the protocol for transient transfections. The mutations that were classified as pathogenic mutations or unclassified variants in our previous reports, have been further investigated. As positive controls, four pathogenic mutations (p.Phe107del, p.Tyr317X, p.Asn336del, p.Ile347del), based on their deleterious character (deletion, nonsense mutation) were transiently transfected. Indeed, overexpression of any of these variants did not restore Cr uptake in SLC6A8 deficient primary fibroblasts (range 7.0 - 7.9 pmol Cr/µg protein; Figure 1). Conversely, transient transfection of the wild-type SLC6A8 ORF restored Cr uptake to normal levels (29.3 pmol Cr/µg protein; Figure 1). In addition, the p.Met560Val variant, originally identified in a patient from the European XLMR panel [7], but more recently found in a healthy control male

[12], could be used to validate our diagnostic approach, since overexpression of this variant resulted in similar uptake activity as observed in the wild-type transfectants (31.6 pmol Cr/µg protein; Figure 1).

Overexpression of five missense variants (p.Gly87Arg, p.Cys337Trp, p.Pro390Leu, p.Arg391Trp and p.Pro554Leu) did not result in a significant increase of Cr uptake (4.9 – 8.6 pmol Cr/µg protein: Figure 1). Overexpression of the p.Val629Ile allele results in a six-fold increase of Cr uptake (29.8 pmol Cr/µg protein), which is similar to the Cr uptake detected in wild-type and p.Met560Val transfectants. Therefore, this variant has now been proven to be a non-pathogenic variant. Identical conclusions could be drawn for the p.Lys4Arg and p.Gly26Arg alleles (23.5 and 44.1 pmol Cr/µg protein).

Previously, we have formulated a list of criteria to classify novel variants in the coding region of *SLC6A8*. These criteria are most needed for the classification of missense variants that were not previously proven to be pathogenic. First, a variant should not be detected in at least 210 male control chromosomes, which reduces the chance of being a polymorphism to less than 1% [20]. Second, if applicable, the variant should cosegregate with the disease (e.g. mental retardation). It should be noted that the inheritance pattern may suggest that the variant is the disease causing mutation, while in fact it may be a variant on the same allele. Thus, absence of cosegregation rules out pathogenicity of the variant, while cosegregation rather gives an indication of probability. Third, high conservation within the SLC6 family of the affected amino acid is a strong suggestion for its importance in protein function. If the affected amino acid is not or less conserved, this suggestion becomes less strong. Additional properties strengthen the probability of pathogenicity, such as the difference in chemical properties of the substituted amino acid compared to the original amino acid, e.g. basic/acidic, aliphatic/aromatic, uncharged/charged, big and rigid/small and flexible. The SLC6A8 N-terminal and C-terminal ends are not conserved. Hence, in these regions, only the chemical

properties can be evaluated properly. However, little is known about the function of these regions (e.g. stability or trafficking of the protein).

A combination of these criteria has been advocated not only for *SLC6A8* mutations, but also for other genes such as *MECP2* (a XLMR gene) [21] and *BRCA*1/2 (breast cancer susceptibility genes) [22], and are often the only tools available if no functional assays are available. The presumed pathogenic nature of variants found in our previous studies, could now be studied by functional assays. This approach resulted in identical classification of DNA variants as the overexpression model. However, in case of prenatal diagnosis, the overexpression model, is essential for unequivocal classification of novel exonic sequence variants.

We and others have shown previously that the prevalence of SLC6A8 deficiency is relatively high in patient panels with mental retardation of unknown etiology as well as in a panel of XLMR males [7,12,14]. This justifies inclusion of SLC6A8 deficiency in the routine testing in these male patients. The diagnostic approach depends on the available facilities and the cooperation of the patients and their guardians. For initial testing, three tests are available that each have respective advantages: 1) brain MRS, 2) urine metabolite testing and 3) DNA analysis (Figure 2). The DNA analysis can result in three options (Figure 3): a pathogenic mutation is found, b) no mutation is found, or c) a novel variant is detected. Following the guidance of the flowcharts facilitates a conclusive diagnosis (or exclusion) of either SLC6A8 deficiency or a Cr biosynthesis defect.

In summary, the Cr uptake assay in primary fibroblasts is a valuable tool for proper diagnosis (or ruling out) of SLC6A8 deficiency, whereas overexpression of *SLC6A8* alleles containing missense variants, to be investigated in SLC6A8 deficient cells, is a valid tool for unequivocal classification of these variants. By applying these studies, we have shown that nine alleles

proved not to result in Cr uptake activity in SLC6A8 deficient fibroblasts upon transient transfection, and thus could be classified as pathogenic mutations, and five alleles were proven to be rare variants that have Cr uptake activity similar to the wild-type SLC6A8. In case prenatal diagnosis is required it is essential to prove pathogenicity of the missense variant by overexpression studies (e.g. transient transfection into SLC6A8 deficient cells).

Table 1. Reference values for the uptake assay in fibroblasts measured by SID-GCMS.

	25µM Creatine	500µM Creatine	500µM Creatine + 500µM GPA
Control (n=13)	27.8 ± 5.6	41.3 ± 7.0	17.7 ± 4.4
range	18.8 – 38.0	31.9 – 51.7	13.0 – 29.0
Inter-assay (n=5)	27.8 ± 3.2		
Intra-assay (n=10)	27.1 ± 1.9		
Deficient (n=12)	0.58 ± 1.03	6.22 ± 2.14	4.58 ± 1.79
range	0.0 – 3.16	2.1 – 10.54	3.16 – 6.76

Table 2. Features of presumed pathogenic mutations and coding unclassified variants in *SLC6A8 [7,12])*.

Mutation	Conserved	Segregation	Urine	Uptake assay	H-MRS
p.Lys4Arg[b]	-	NA	NA	NA	NA
p.Gly26Arg[b]	Species	NA	NA	NA	NA
p.Gly87Arg[a]	+	NA	NA	NA	NA
p.Phe107del[a]	+	+	+[c]	+[c]	NA
p.Tyr317X[a]		NA	NA	NA	NA
p.Asn336del[b]	+	NA	NA	NA	NA
p.Cys337Trp[a]	+	+	+	NA	NA
p.Ile347del[b]	Functional	NA	NA	NA	NA
p.Pro390Leu[a]	+	+	NA	+[c]	NA
p.Arg391Trp[b]	Region	NA	NA	NA	NA
p.Pro554Leu[a]	+	+	+	+[d]	NA
p.Met560Val[a]	-	+	NA	-	Normal
p.Val629Ile[a]	Species	+	-	NA	NA

[a] [7], [b] [12], [c]Performed in cells of an unrelated patient with the same mutation, [d]Became available after publication.
NA= not available

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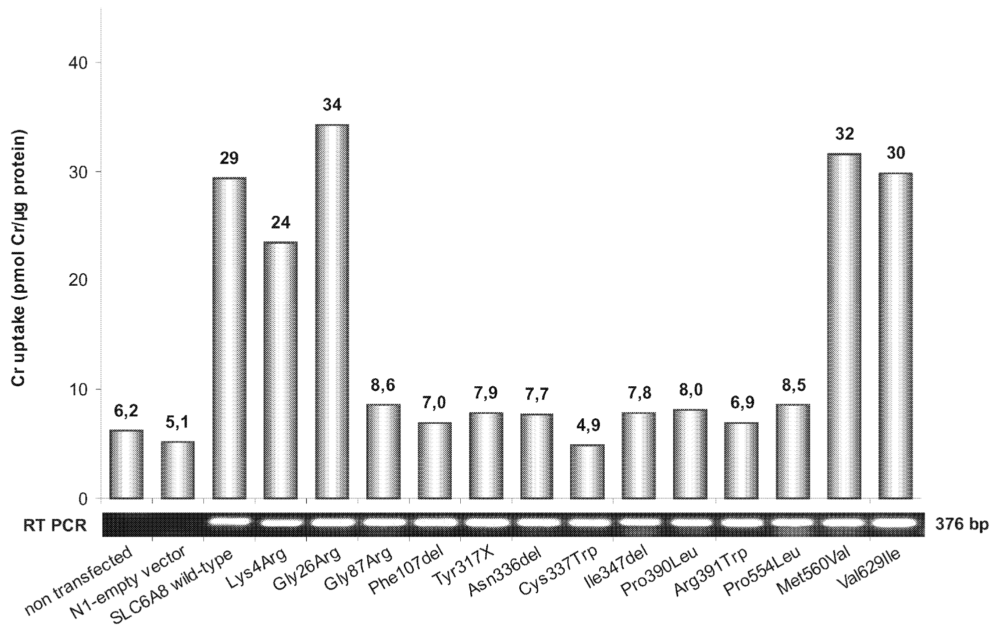
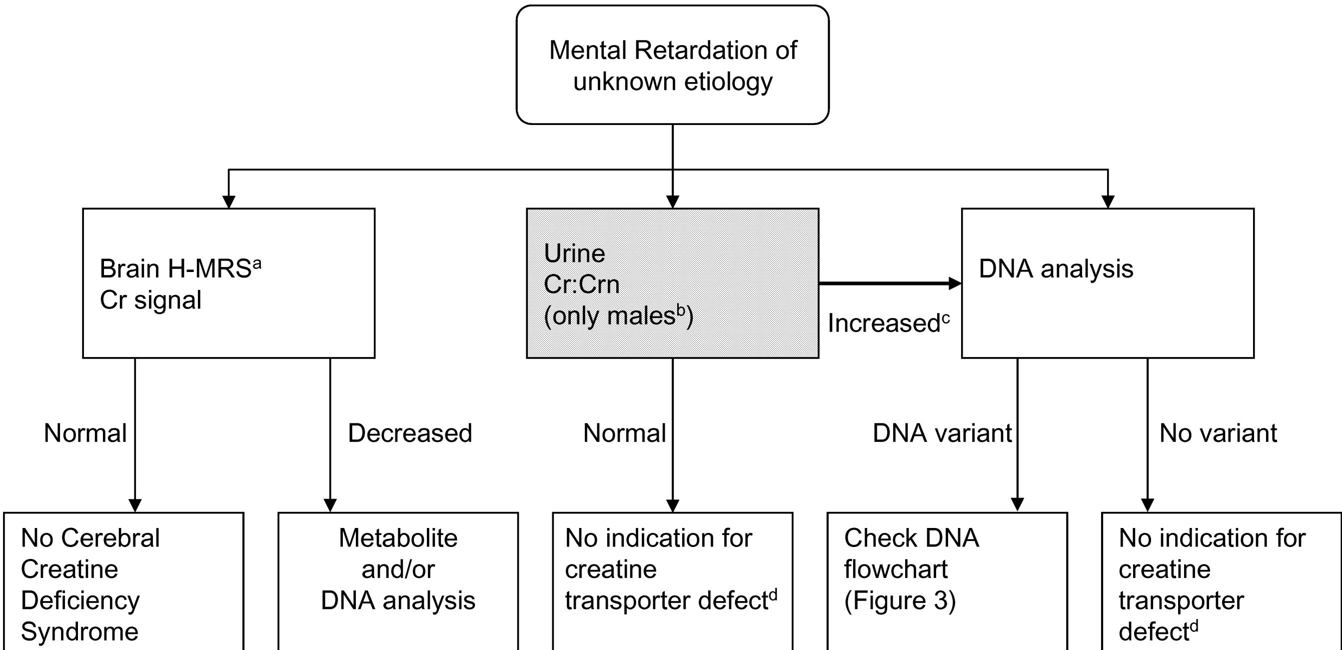


Figure 1. Creatine uptake in SLC6A8 deficient fibroblasts, transiently transfected with SLC6A8 variants. Results are representative for the triplo measurements after 24 hours with 500 µM Cr. SLC6A8-EGFP mRNA expression is confirmed by RT-PCR. In contrast to the mock and non-transfected cells, expression of SLC6A8-EGFP mRNA was observed in all cells transfected with wild-type or variant pEGFP-SLC6A8 constructs.



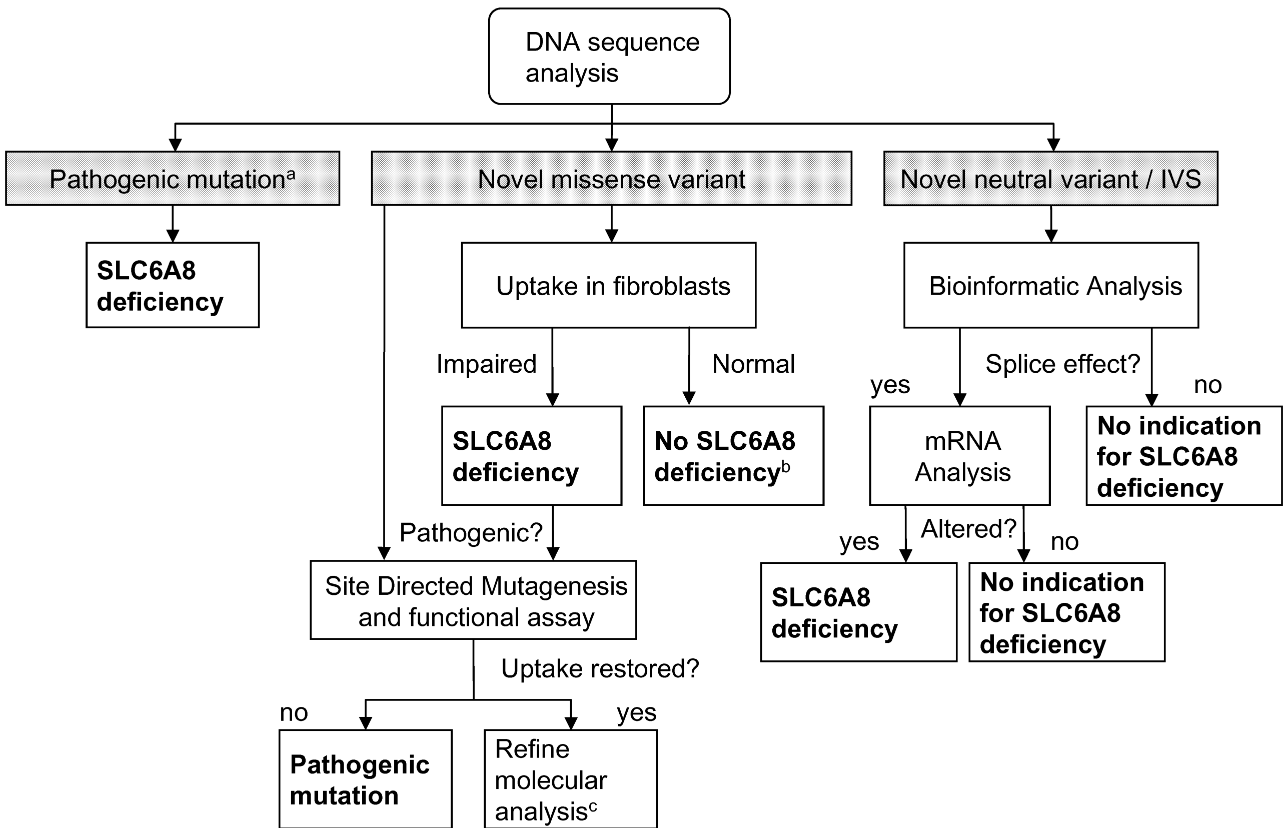
[a] Brain MRI/H-MRS may result in detection of an other disorder.
[b] Usually, in females the Cr:Crn ratio is not informative.
[c] In approximately 10% a false positive Cr:Crn ratio is found.
[d] There is no information on the frequency of false negative results in metabolite and DNA analysis.

Figure 2. Schematic diagram for screening for SLC6A8 deficiency in patients with mental retardation of unknown etiology.

Brain H-MRS A highly decreased Cr signal in brain H-MRS is a strong indication for primary cerebral creatine deficiency syndrome. In the majority of the cases, this is caused by SLC6A8 deficiency. However, this may also be the result of a Cr biosynthesis defect. Thus, metabolite and/or DNA analysis is warranted for a definitive diagnosis. The advantage of brain MRI/MRS is that other disorders can be detected simultaneously [14]. However, MRS is only available in specialised institutes and the mentally retarded patient needs to be sedated.

Urine Cr:Crn An elevated urinary Cr:Crn ratio is a strong indication for SLC6A8 deficiency [17]. In that case, DNA analysis is warranted to confirm the diagnosis. Furthermore, urine metabolite analysis may also reveal a Cr biosynthesis defect especially if guanidinoacetic acid, the precursor of creatine is included [17]. Our experience is, that in about 10% of the patients with elevated urinary Cr:Crn ratio, no DNA variant can be detected (data not shown). These cases likely represent false positives, as a secondary effect of another disease.

DNA analysis DNA or blood is available from most patients for routine testing of common causes of mental retardation. Therefore, genomic testing for SLC6A8 deficiency will not put additional burden on the patient. Furthermore, in many cases the result of DNA analysis can provide the definitive diagnosis of SLC6A8 deficiency. The DNA analysis can result in three options (see figure 3).



[a] A mutation is considered to be pathogenic if it 1) is a nonsense mutation, 2) is a deletion, 3) causes a frameshift, 4) causes aberrant splicing or 5) has been proven to be pathogenic, previously.
[b] In case Cr signal is reduced on brain H-MRS, AGAT and GAMT-deficiency need to be investigated.
[c] If brain H-MRS and/or uptake in fibroblasts indicate SLC6A8 deficiency, but site directed mutagenesis classifies the missense variant as a polymorphism, additional molecular analysis should be performed to identify the pathogenic mutation.

Figure 3. Decision flowchart for molecular investigations of *SLC6A8*.

A **pathogenic mutation** is found, proving SLC6A8 deficiency in the patient. A mutation is considered pathogenic if it is a nonsense mutation, a deletion, a frameshift mutation, a splice error or previously published and well-described as pathogenic. In such a case, SLC6A8-deficiency is sufficiently proven and further work up is not necessary. A **novel missense variant** may be found that was not reported before, nor was it found in male control DNA samples. Then, Cr uptake assay in fibroblasts should be performed. A normal Cr uptake profile in fibroblasts excludes SLC6A8 deficiency, and the patient should be tested for GAMT and AGAT deficiency in case the Cr signal in brain H-MRS was reduced. If, however, a Cr uptake assay could not be performed, or the fibroblasts prove to be deficient in Cr uptake, further studies are needed to prove that the variant is the pathogenic mutation. Hereto, overexpression of the variant, as described in the present study, should be performed. If overexpression of the variant does not restore Cr uptake in the SLC6A8 deficient host cells, it is a pathogenic mutation. If, however, Cr uptake is restored, then the variant represents a non-pathogenic variant and molecular analysis should be refined by *SLC6A8* mRNA analysis.

A **novel intronic (IVS) or translational silent variant** is detected that was not reported before, nor was it found in male control DNA samples. In that case, bioinformatic analysis (e.g. Fruitfly, Splicepredictor, NetGene2, ESEfinder; www.creatinedeficiency.info/webtools) can be used to predict possible aberrant splicing. If no effect on mRNA splicing is predicted, the variant likely represents a non-pathogenic variant and there is no indication for SLC6A8 deficiency. However, if bioinformatic analysis reveals a possible effect on splicing, SLC6A8 mRNA analysis is needed. In case alternative splice variants are detected, SLC6A8 deficiency is confirmed.

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Chapter 6

Progressive intestinal, neurological and psychiatric problems in two adult males with cerebral creatine deficiency caused by an *SLC6A8* mutation.

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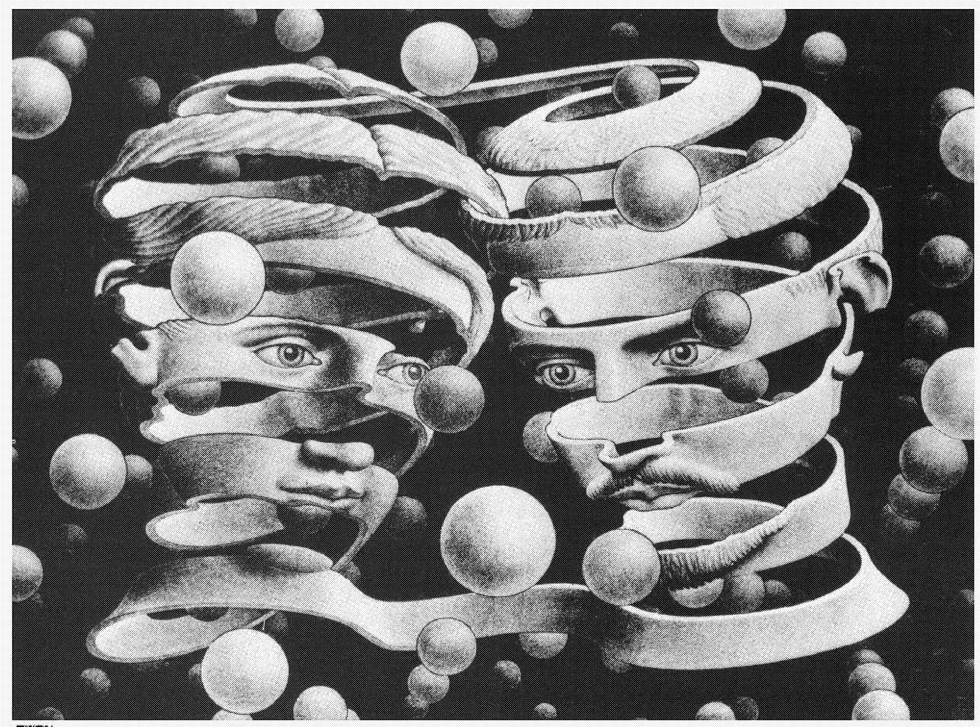

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Letter to the Editor

Progressive intestinal, neurological and psychiatric problems in two adult males with cerebral creatine deficiency caused by an *SLC6A8* mutation

To the Editor:
Recently, an XLMR syndrome was identified, comprising mental retardation, low weight and poor muscle build, autistic behaviour, epilepsy, and expressive speech and language delay, caused by cerebral creatine deficiency due to mutations in the creatine transporter gene *SLC6A8* (1–9). Additionally, an impaired uptake of creatine in fibroblasts, increased levels of creatine : creatinine ratio in urine and a marked reduction of creatine signal in the H-MRS of brain were seen (8).

We report the characteristics of two additional patients identified previously in the screening of the *SLC6A8* gene in a cohort of 288 male patients (9) and compare the clinical phenotype to other published cases (Table 1) (Fig. 1).

Case 1 (N87) was the oldest son born to healthy non-consanguineous parents with severe mental retardation. He worked in a sheltered environment until the age of 57 years when he was institutionalized. Neurological examination at the age of 70 years showed a myopathic face with ptosis, external ophthalmoplegia and open, hanging mouth. CPK and TSH levels were normal.

Case 2 (N87), the younger brother of case 1, attended special education and learnt to write and read. He worked in a sheltered environment. After the death of his father when case 2 was 51 years old, severe regression in cognitive (total IQ was 50 before and 34 a few years after this event) and social functions occurred, believed to be the result of chronic mood disturbance. At the age of 52, he became institutionalized and continued to have depressions. In his fifties, he had urological problems (urethra stenosis) and gastrointestinal problems (chronic constipation and a bowel ileus). Spontaneous luxations of several digits had occurred since the age of 50. Neurological examination at the age of 67 showed Parkinsonism (probably caused by medication), upward gaze paresis, expressionless face, hanging mouth and hanging shoulders. However, no additional signs of myopathy were detected by CPK analysis and EMG studies. A cerebral CT scan showed no abnormalities.

Photographs at younger and older age clearly underlines the progressiveness in the clinical features in this patient.

The sister of cases 1 and 2, aged 59 years, was a mutation carrier. She had a short stature (154 cm; −2.5 SD) and learning difficulties. Since the age of 55, she had a severe constipation requiring surgical intervention. She had one healthy son.

So far, only one other family with elderly patients has been described (5). Features present in these adult patients are myopathic facies (including ptosis), hyperextensible joints, soft skin and gastrointestinal problems, such as megacolon, severe constipation and bowel ileus. The latter might be a consequence of (smooth) muscle problems or autonomic nerve dysfunction. The difference in the characteristics present in the older compared to the younger patients might be explained by a selection bias in the identification of the latter. With the exception of the two families of Mancini et al. (6), all younger patients underwent H-MRS of the brain as part of a diagnostic work-up because of encephalopathy, speech problems and seizures and all were diagnosed by the absence of creatine on this spectroscopy. The older patients were identified through candidate gene analysis (5) and by screening of the *SLC6A8* gene in an XLMR cohort, without prior knowledge of phenotypic characteristics. In addition, clinical examination was not performed extensively in all previously reported patients, which might lead to under-recognition of several

Table 1. Summary of clinical symptoms in male patients with *SLC6A8* mutations

Features	Previous reports								Present family		Total (%)
	Cecil et al. (2)[a]	Hahn et al. (5)	DeGrauw et al. (3)		Bizzi et al. (1)		Mancini et al. (6)				
General											
Number of patients (per family)	1	5	2	1	1	1	2	2	Case 1	Case 2	17
Age at examination (years)	6	15–66	17–20	4	2	5	6–8	8–10	68	64	
Neurological											
Degree of mental retardation	Moderate–severe	severe (5/5)	severe	NA	mild	NA	moderate	moderate	severe	severe	mild–severe
Expressive language difficulties	+	5/5	2	+	+	+	2	2	+	+	17/17 (100)
Hypotonia	+	2/2	–	–	–	–	2	2	+	+	10/14 (71)
Seizures	+	5/5	2	–	–	+	–	–	–	–	9/17 (53)
Movement disorder; spastic/dystonic/ataxic	–	3/5	1	–	–	–	2	2	–	–	8/17 (47)
Physical examination											
Short stature	–	–	2	–	–	–	2	–	+	+	7/17 (41)
Low weight	–	–	–	–	–	–	2	2	–	+	5/17 (29)
Microcephaly	–	–	1	–	–	–	–	–	–	–	1/17 (6)
Midface hypoplasia/flat malar area	–	3/3[b]	–	–	–	–	–	2	–	+	6/15 (40)
Myopathic facies	–	1/3[b]	–	–	–	–	–	–	–	+	2/15 (13)
Ptosis	–	1/3[b]	–	–	–	–	–	–	+	+	3/15 (20)
Stub thumb	–	3/3[b]	–	–	–	–	–	–	–	–	3/15 (20)
Simian crease	–	–	–	–	–	–	–	–	–	+	1/17 (6)
Soft skin	–	3/3[b]	–	–	–	–	–	–	–	+	4/15 (27)
Hyperextensible joints	–	3/3[b]	–	–	–	–	–		–	+	4/15 (27)
Other											
Gastrointestinal problems	–	4/5[b]	–	–	–	–	–	–	+	+	6/17 (35)
Behaviour disturbance/mood disorder	–	5/5	1	–	–	+	2	2	–	+	12/17 (71)
Laboratory investigations											
Increased urine creatine : creatinine ratio	+	2/2	+	+	+	+	1/1	+	+	+	12/12 (100)
Impaired creatine uptake fibroblasts	+	2/2	+	+	NA	+	2	2	NA	NA	10/10 (100)
Absence/reduced creatine on H-MRS	+	NA	2	+	+	+	2	NA	NA	NA	8/8 (100)
Mutation in *SLC6A8*	R514X	G381R	F107del	F408del	Y262X	F408del	ivs10 + 5G-T	P544L	C337W		

NA, not applicable.
[a]This patient has also been described in the reports of Salomons et al. 2001 and DeGrauw et al. 2003.
[b]Only present in the affected older males of this family.

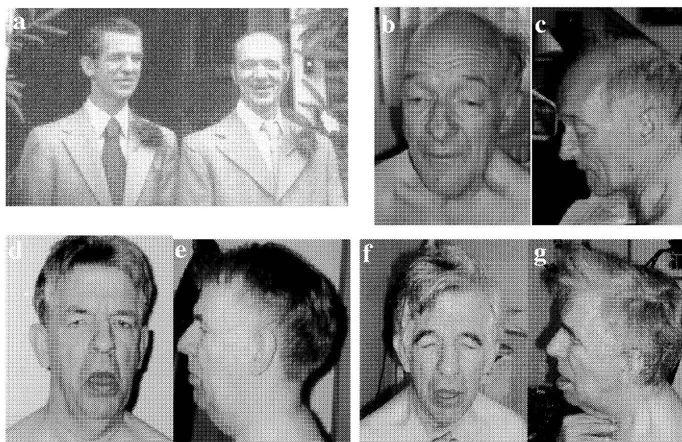

Fig. 1. (a) The brother pair of family 1 between 30 and 40 years old; case 1 on the right, case 2 on the left. (b, c) Case 1 at the age of 68 years. Note ptosis. (d, e) Case 2 at the age of 57 years. (f, g) Case 2 at the age of 64 years. Note the progressiveness in facial features.

symptoms. Another more likely explanation is that the condition is progressive and gives rise to additional features at later age. It is of note that in another report of two brothers progressive atrophy of the brain was showed by image studies (3). Therefore, clinical follow-up should give more insight into the phenotypic outcome at later age.

The female carrier presented did have learning difficulties, which is in line with previous reports (8). She experienced the same severe bowel problems as her brother, which might well reflect a symptom of creatine transporter deficiency.

In conclusion, the clinical presentation of SLC6A8 deficiency is broader than initial appreciated and it might include the progression of intestinal, neurological and psychiatric problems later in adult life.

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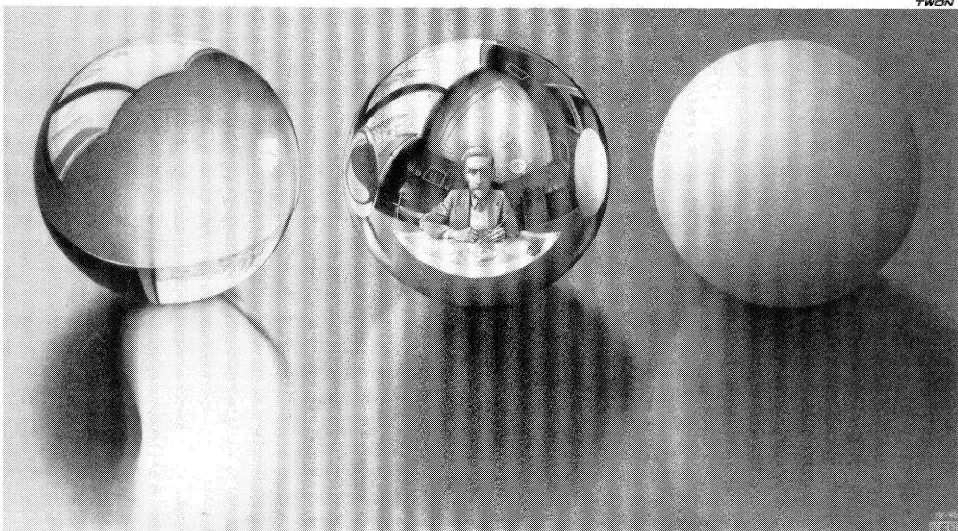
Chapter 7

Are cerebral creatine deficiency syndromes on the radar screen?

*Future Neurology (2006) **1**(5), 637-649, invited review*



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Are cerebral creatine deficiency syndromes on the radar screen?

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Cerebral creatine deficiency syndromes (CCDS) are responsible for a considerable proportion of the population affected with mental retardation. CCDS are caused by either an inborn error of the proteins involved in creatine biosynthesis or in the creatine transporter. Besides mental retardation, the clinical characteristics of CCDS are speech and language delay, epilepsy and features of autism. CCDS can be diagnosed by proton magnetic resonance spectroscopy of the brain and/or by biochemical and molecular analysis. Treatment of the defects in creatine biosynthesis has yielded favorable outcomes, while treatments for creatine transporter deficiency are still under investigation at this time. The relatively large contribution of the CCDS to the monogenic causes of mental retardation emphasizes the importance of including CCDS in the differential diagnosis of mental retardation of unknown etiology. Pathophysiology is not yet unravelled, although it is known that creatine plays an important role in energy storage and transmission. Moreover, *in vitro* data indicate that creatine acts as a neuromodulator in the brain.

Keywords: AGAT, cerebral creatine deficiency syndromes, creatine, GAMT, *GATM*, guanidino compounds, mental retardation, metabolic disorders, neuromodulator, SLC6A8, solute carrier family 6 member 8

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In the last decade, a novel group of inborn errors of proteins involved in creatine biosynthesis and its transporter has been identified. The high prevalence of these defects within the mentally retarded population and the promising treatment possibilities argue for the inclusion of cerebral creatine deficiency syndromes (CCDS) in the differential diagnosis of mental retardation of unknown etiology. CCDS arise by mutations in either one of the autosomal genes *AGAT* and *GAMT*, which encode the two enzymes (arginine:glycine amidinotransferase and guanidinoacetate methyltransferase, respectively) [1,2] necessary for creatine biosynthesis, or by mutations in the X chromosomal creatine transporter gene (solute carrier family 6 member 8 [*SLC6A8*]) [3]. Until recently, this group of syndromes has been termed creatine deficiency syndromes (CDS). However, in bodily fluids, no creatine deficiency exists in creatine transporter-deficient patients, thus, this term may be misleading. We therefore prefer to use the term CCDS, which also correlates better to the main clinical hallmarks that are related to CNS involvement.

In the general population, the incidence of mental retardation is estimated to be approximately 1% (profound, severe and moderate cases) to 3% if mild cases are included (IQ 50–70). Within the mentally retarded population, the frequency of X-linked mental retardation (XLMR) is estimated at 5–12% [4]. This number, and the fact that a large group of XLMR genes are already known [5], indicates that each monogenic cause of XLMR only accounts for a small percentage of mental retardation in males [6,7]. For the fragile X mental retardation gene (*FMR1*), the Aristaless-related homeobox gene (*ARX*) and, to a lesser extent, for the creatine transporter gene (*SLC6A8*), this seems to be different, as they account for a larger proportion of the XLMR subgroup [7].

The prevalence of SLC6A8 deficiency has been studied in three different patient groups. In the first panel, consisting of males with a strong predilection to having XLMR, a prevalence of 2.1% (six out of 288) was found. Considering the estimation that only 10% of the patients with mental retardation are affected with an X-linked defect, the prevalence of 2.1% in XLMR would translate to approximately 0.2% in the general mental retardation population of unknown etiology [8]. However, the prevalence in the two panels with mental retardation (four out of 478 = 0.8%, [9]) and global development delay (two out of 92 = 2.2%, [10]) was somewhat higher than predicted. This latter group was investigated by proton magnetic resonance spectroscopy (MRS), whereas the other groups were studied by DNA sequence analysis.

The prevalence of AGAT and GAMT deficiencies are not expected to be high, since they are autosomal recessive disorders. Indeed, only 29 GAMT- and five AGAT-deficient patients have been reported [11–15]. However, awareness of GAMT deficiency may be of utmost importance in Mediterranean countries, because a high carrier rate of a pathogenic *GAMT* mutation exists [16,17], and most of the GAMT-deficient patients are from this region.

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The relatively high prevalence of CCDS highlights the importance of CCDS inclusion in the differential diagnosis of mental retardation of unknown etiology.

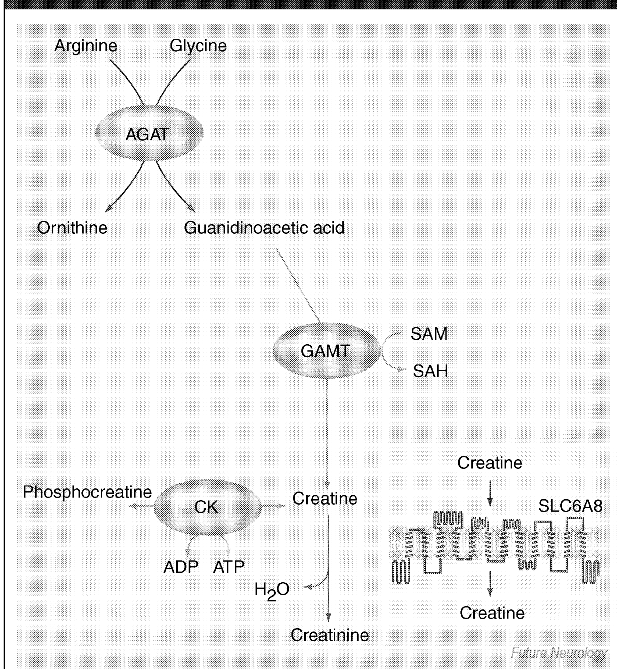
Inborn errors of proteins involved in creatine biosynthesis & its transporter

Arginine:glycine amidinotransferase deficiency

The initial and rate-limiting step in creatine biosynthesis is catalyzed by the AGAT enzyme, which forms guanidinoacetic acid, the precursor of creatine (Figure 1). In 2000, the first siblings with a defect in this enzyme were recognized [18]. These sisters (4- and 6-years old) presented with mental retardation and severe language delay. Routine blood and urine analyses were normal and further investigations did not suggest a neurometabolic disorder. Magnetic resonance imaging (MRI) of the brain was normal, but proton MRS revealed the absence of the creatine–phosphocreatine signal, suggesting a defect in the proteins involved in creatine biosynthesis or in the creatine transporter. In plasma, concentrations of creatine and guanidinoacetic acid were within normal values, which ruled out a deficiency of GAMT. The patients were reinvestigated in 2001, and analysis of urine revealed consistently decreased levels of guanidinoacetic acid [19]. The diagnosis of AGAT deficiency was confirmed by impaired AGAT activity in cultured cells and the detection of a pathogenic mutation in exon 3 of the *AGAT* gene [19].

Presently, five AGAT-deficient patients are known from two unrelated families, in whom reduced levels of GAA in body fluids have been detected [14,15]. The general clinical and biochemical findings are listed in Table 1.

Figure 1. Creatine biosynthesis.



Creatine biosynthesis involves a two-step reaction: the first is catalyzed by arginine:glycine amidinotransferase (AGAT, EC 2.1.4.1) and the second by guanidinoacetate methyltransferase (GAMT, EC 2.1.1.2). Creatine is transported via the bloodstream and taken up by tissues with high CK activity, such as muscle and brain, via a creatine transporter (SLC6A8). CK catalyzes the phosphorylation and dephosphorylation of creatine and phosphocreatine, respectively, thus providing a high-energy phosphate buffering system during ATP release and use.
ADP: Adenosine diphosphate; ATP: Adenosine triphosphate; CK: Creatine kinase.

Guanidinoacetate methyltransferase deficiency

The second step in creatine biosynthesis is mediated by the GAMT enzyme (Figure 1). In 1994, the first GAMT-deficient patient, a 22-month-old male was identified by Stöckler and collaborators [20,21]. The onset of symptoms started at 5 months with developmental arrest. The patient was hypotonic, unable to sit or roll over, showed uncoordinated swallowing and developed a severe extrapyramidal disorder. He had no organomegaly and his head circumference, hearing and vision appeared normal. Electrocardiogram and cardiac ultrasound examinations were normal; electroencephalogram showed low background activity and multifocal spikes. Nonspecific biochemical elevations were reported due to decreased creatinine levels. Magnetic resonance studies were performed for the first time at the age of 12 months: MRI revealed bilateral abnormalities in the globus pallidus. Proton MRS showed a spectrum lacking a creatine signal and an elevated guanidinoacetic acid peak. Treatment with arginine, the substrate of the AGAT enzyme, did not result in restoration of brain creatine. Moreover, guanidinoacetic acid in the brain, measured by proton MRS, remained increased, indicating that the defect was caused by a block in GAMT activity [20]. Indeed, in 1996, impaired GAMT activity in cultured cells and pathogenic mutations in the *GAMT* gene were identified [21]. Furthermore, a GAMT knockout mouse model has been developed, mimicking the biochemical characteristics of GAMT deficiency in humans [22–24].

Table 1. Overview of the patients described to date.

Cerebral creatine deficiency	*AGAT* (*n* = 5)§	*GAMT* (*n* = 29)¶	*SLC6A8* (*n* = 24)#
Clinical traits:			
Age at diagnosis	0–5	0–26	2–66 years
Developmental delay	5/5	27/29	24/24
Speech and language delay	5/5	21/29	24/24
Mental retardation	5/5	27/29	24/24
Hypotonia	2/5	27/29	11/16
Behavior disorder	1/5	21/29	14/19
Movement disorder	NR	15/29	10/19
Seizures	NR‡	25/29	16/24
Mild phenotype*	NA	3/29	NA
Intermediate phenotype*	NA	12/29	NA
Severe phenotype*	NA	12/29	NA
H-MRS of the brain: absence/reduction Cr	5/5	27/29	12/12
Biochemical findings:			
Urinary creatine:creatinine			Increase in 17/17
Urinary guanidinoacetic acid	Decrease in 5/5	Increase in 29/29	Increase in 2/4
Plasmatic guanidinoacetic acid	Decrease in 5/5	Increase in 28/28	Increase in 1/2
Plasmatic creatine	Decrease in 5/5	Decrease in 28/28	NR
Treatment available	Yes	Yes	?

**Based on [11].*
‡Febrile seizures were reported in one of the first patients to be described [18].
§ [14,15,19]; ¶ [11,12,13]; #[9,53,66,67].
AGAT: Arginine–Glycine amidinotransferase; GAMT: Guanidinoacetate methyltransferase; NA: Not applicable; NR: Not reported.

To date, 29 patients have been described, varying from neonate to 29 years of age [11–13]. Owing to heterogeneous clinical presentation, GAMT-deficient patients can be classified as having a mild, moderate or severe phenotype, based on the severity grade of the main clinical characteristics (i.e., mental retardation, epilepsy and movement disorder). An overview of the clinical characteristics reported so far is presented in Table 1.

Creatine transporter deficiency

The creatine transporter, encoded by the *SLC6A8* gene, is essential for creatine uptake into cells. In 2001, the first patient with SLC6A8 deficiency was described. The patient presented with mild developmental delay at the age of 7 months in combination with central hypotonia. Prenatal and perinatal histories were unremarkable [25]. There was a history of learning disabilities and mental retardation in the family, compatible with an X-linked disorder. At 2 years of age the patient was admitted to hospital owing to a partial status epilepticus. Examination at 6 years of age showed a severe delay in speech and language development. A progressive increase of the head circumference (75th percentile to 95th percentile) prompted MRI and proton MRS. Proton MRS highlighted an almost complete loss of the creatine and phosphocreatine signal, similar to that observed in patients with AGAT and GAMT deficiency [26]. Consequently, oral creatine supplementation was commenced. However, after 4 months no restoration of cerebral creatine concentration was observed, which was in line with the lack of clinical improvement. Therefore, oral creatine supplementation was discontinued.

This, and the biochemical findings, ruled out a creatine biosynthesis defect. Moreover, high urinary creatine concentrations suggested a defect in cellular creatine transport. The inheritance pattern suggestive for X-linked disease and the fact that the gene encoding the creatine transporter, *SLC6A8*, is mapped to Xq28 [27], strengthened this hypothesis. This transporter is a member of the Na^+/Cl^--dependent neurotransmitter transporter family (Figure 2). Indeed, sequence analysis of *SLC6A8* revealed a hemizygous nonsense mutation. Furthermore, impaired creatine uptake in cultured fibroblasts was demonstrated [28]. The carrier status for this mutation was confirmed in the female relatives.

So far, more than 34 patients have been reported with SLC6A8 deficiency. The clinical characteristics of 24 male patients are listed in Table 1. In females, a very heterogeneous clinical phenotype is expected due to skewed X-inactivation, varying from learning disabilities to mental retardation.

How to diagnose cerebral creatine deficiency syndromes?

A marked reduction of cerebral creatine measured by proton MRS is highly indicative of a primary creatine deficiency syndrome. Workup by metabolite, molecular and functional studies is required to identify the underlying defect (i.e., AGAT, GAMT or SLC6A8 deficiency). Additionally, in families where individuals have previously been diagnosed with CCDS, prenatal diagnosis can be performed at the molecular level in chorion villus sampling or amniotic cells [12,15]. In case of GAMT deficiency, prenatal diagnosis can also be performed from the amniotic fluid at the metabolite level [12]. At present, the primary choice for

Figure 2. Schematic representation of *SLC6A8* with the putative 12 transmembrane domain structure.

Published single amino acid mutations are depicted as blue squares. Cys144 is marked as a green hexagon [62]. Possible *N*-glycosylation substrates N192, N197 and N548 are depicted as black hexagons. The putative Leu-zipper (L286, L293, L300 and I307) is marked with purple upwards arrows. Putative cyclic adenosine monophosphate phosphokinase phosphorylation sites T56 and S256 are shown as orange downwards arrows. The figure was plotted with TOPO2 (Johns SJ, TOPO2, Transmembrane protein display software [101]).

screening of CCDS in most institutes is measuring creatine, creatine:creatinine ratio and guanidinoacetic acid in body fluids or performing proton MRS of the brain. In contrast with metabolite analysis, proton MRS is expensive, not widely available and sedation is usually required.

Metabolite analysis

Various methods to measure creatine and guanidinoacetic acid in bodily fluids are available [29]. In diagnostic settings, the techniques generally used are gas chromatography-mass spectrometry [30,31], high-performance liquid chromatography (HPLC) [32] and tandem mass spectrometry [33–35]. Increased guanidinoacetic acid levels in bodily fluids are pathognomonic for GAMT deficiency, whereas reduced levels are found in AGAT deficiency. Creatine is usually reduced in the bodily fluids of patients with a biosynthesis defect, whereas an increased urinary creatine:creatinine ratio is found in males with SLC6A8 deficiency [30]. In the majority of females with a heterozygous *SLC6A8* mutation, this ratio is not informative.

Molecular diagnosis

Molecular diagnosis is usually performed by direct sequencing of the open reading frame of the respective gene or by mutation scanning using DHPLC [36] followed by DNA sequence analysis in case of aberrant heteroduplexes.

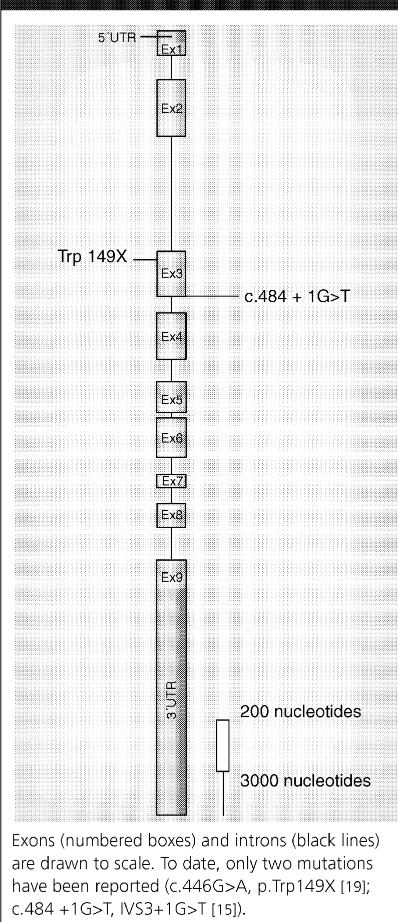
- The *AGAT* gene (Gene ID 2628, official nomenclature: *GATM*) has been mapped to chromosome 15q15.3, is 16.8 Kb in size and contains nine exons, which encode a protein of 424 amino acids. There are two mutations described (Figure 3);
- The *GAMT* gene (Gene ID 2593) has been mapped to chromosome 19p13.3, its size is 4.46 Kb and it contains six exons, which encode a protein of 237 amino acids. There are 15 pathogenic mutations described throughout the gene (Figure 4);
- The creatine transporter gene – *SLC6A8* (Gene ID 6535) has been mapped to Xq28 [37–39]. The *SLC6A8* gene spans 8.4 Kb consisting of 13 exons, which encode a protein of 635 amino acids. To date, 20 mutations have been described throughout *SLC6A8* (Figure 5).

Enzyme analysis

Several assays have been reported to assess both AGAT and GAMT activity [29]. The first methods to be reported used radioactive-labeled substrates [19,21,40,41]. In 2003, two methods using stable isotope labeled substrates were developed for both AGAT and GAMT [42,43]. The use of stable isotopes increases the sensitivity of the methods and reduces the amount of biological material required.

To study SLC6A8 deficiency, the creatine transporter function can be tested by a functional assay in fibroblasts [28].

Figure 3. Schematic representation of the *AGAT* gene and its reported mutations.

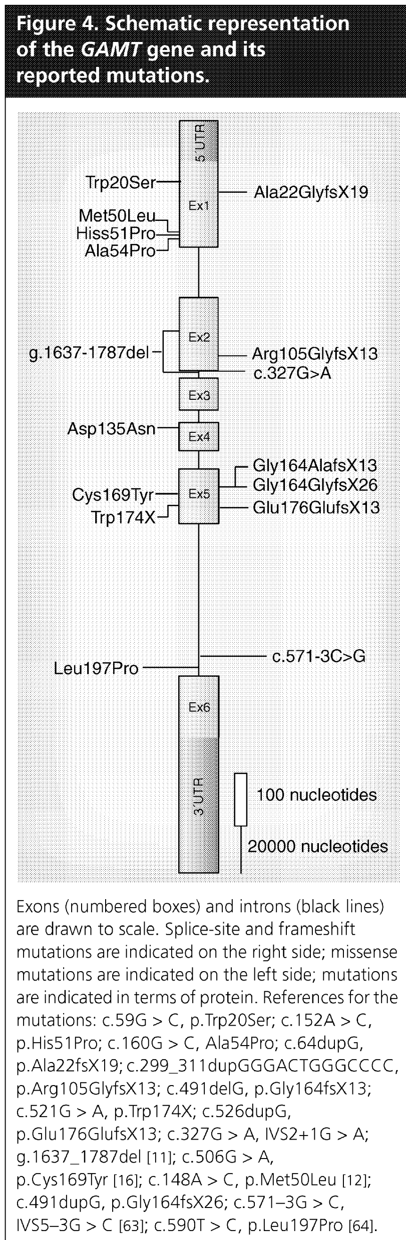


Exons (numbered boxes) and introns (black lines) are drawn to scale. To date, only two mutations have been reported (c.446G>A, p.Trp149X [19]; c.484 +1G>T, IVS3+1G>T [15]).

Diagnostic pitfalls

In the last decade, only two families with AGAT deficiency were reported, in comparison with 22 families affected with GAMT deficiency. AGAT-deficient patients could remain unnoticed

Figure 4. Schematic representation of the *GAMT* gene and its reported mutations.



Exons (numbered boxes) and introns (black lines) are drawn to scale. Splice-site and frameshift mutations are indicated on the right side; missense mutations are indicated on the left side; mutations are indicated in terms of protein. References for the mutations: c.59G > C, p.Trp20Ser; c.152A > C, p.His51Pro; c.160G > C, Ala54Pro; c.64dupG, p.Ala22fsX19; c.299_311dupGGGACTGGGCCCC, p.Arg105GlyfsX13; c.491delG, p.Gly164fsX13; c.521G > A, p.Trp174X; c.526dupG, p.Glu176GlufsX13; c.327G > A, IVS2+1G > A; g.1637_1787del [11]; c.506G > A, p.Cys169Tyr [16]; c.148A > C, p.Met50Leu [12]; c.491dupG, p.Gly164fsX26; c.571–3G > C, IVS5–3G > C [63]; c.590T > C, p.Leu197Pro [64].

in metabolic screening due to techniques that are neither sensitive or specific enough to detect decreased metabolite levels. Furthermore, intake of nutrition containing high concentrations of creatine (i.e., meat and fish) may hamper diagnosis of AGAT deficiency [44].

In approximately 10% of the males with mental retardation in whom elevated urinary creatine:creatinine ratio is found, no *SLC6A8* mutation is detected. They are likely to represent false-positive outcomes. Elevated urinary creatine:creatinine ratio may also be the secondary result of other (neuro)muscular disorders, such as Duchenne muscular dystrophy and Becker muscular dystrophy [45]. In rare cases, promoter mutations or deep intronic mutations may have been missed.

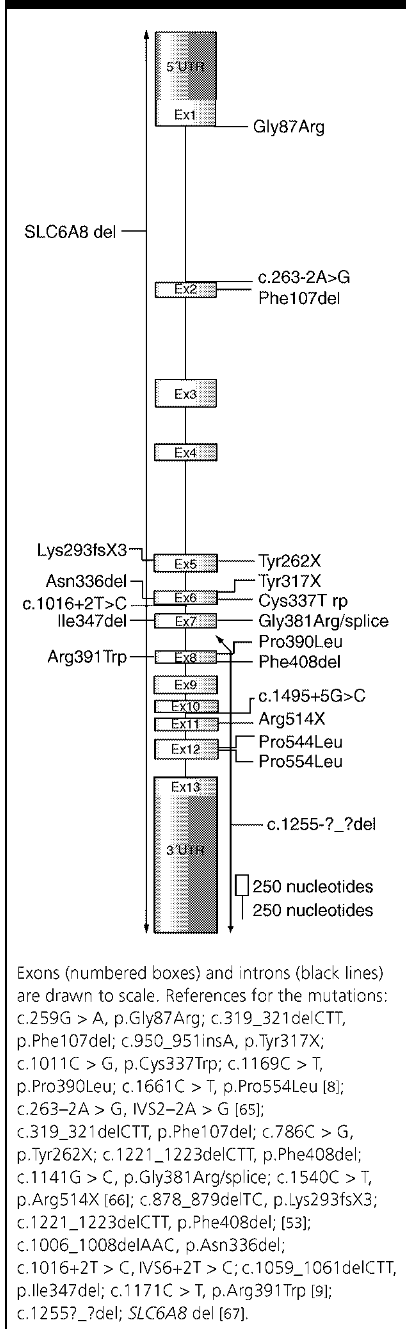
Although three female SLC6A8-deficient patients were found with an elevated urinary creatine:creatinine ratio, this is not the rule for the majority of heterozygous females [29]. Proton MRS of the brain in females may be restricted by difficulties in detecting minimal reductions in brain creatine and sedation is often required. Currently, in females, molecular screening is probably the most sensitive method for the detection of SLC6A8 deficiency, and thus urinary creatine:creatinine ratio is not the primary choice. In molecular diagnostic screening, care should be taken, since a paralogous gene, *SLC6A10*, which is mapped at chromosome 16p11, is highly identical to *SLC6A8* [46].

Regarding DNA sequence analysis, it should be noted that, in rare cases, unique sequence variations are detected, both in coding and noncoding regions in the *SLC6A8*, *GAMT* or *AGAT* gene, which were not encountered in controls [8,9,16]. Thus, it is important to evaluate the nature of the variants carefully, before classifying them as pathogenic mutations or polymorphisms.

Treatment of creatine biosynthesis defects & creatine transporter defects

Treatment approaches of CCDS aim to restore creatine in the brain by creatine supplementation, with success for the biosynthesis defects [20,47,48]. For AGAT deficiency, the highest success rate is expected because there is no accumulation of substrates. In fact, if started in a presymptomatic phase, creatine supplementation in AGAT and GAMT-deficient patients leads to an impressive restoration of cerebral creatine levels, and a favorable clinical response. In an AGAT-deficient infant, treatment initiated at 4 months of age showed normal development by 18 months of age, and the restoration of creatine levels in bodily fluids and the brain were almost complete [49]. Similar success was observed with a GAMT-deficient patient in whom presymptomatic treatment was initiated at 22 days of age [13]. This suggests that creatine supplementation in early life prevents the neurological sequelae.

Figure 5. Schematic representation of the *SLC6A8* gene and its reported mutations.



Exons (numbered boxes) and introns (black lines) are drawn to scale. References for the mutations: c.259G > A, p.Gly87Arg; c.319_321delCTT, p.Phe107del; c.950_951insA, p.Tyr317X; c.1011C > G, p.Cys337Trp; c.1169C > T, p.Pro390Leu; c.1661C > T, p.Pro554Leu [8]; c.263–2A > G, IVS2–2A > G [65]; c.319_321delCTT, p.Phe107del; c.786C > G, p.Tyr262X; c.1221_1223delCTT, p.Phe408del; c.1141G > C, p.Gly381Arg/splice; c.1540C > T, p.Arg514X [66]; c.878_879delTC, p.Lys293fsX3; c.1221_1223delCTT, p.Phe408del; [53]; c.1006_1008delAAC, p.Asn336del; c.1016+2T > C, IVS6+2T > C; c.1059_1061delCTT, p.Ile347del; c.1171C > T, p.Arg391Trp [9]; c.1255?_?del; *SLC6A8* del [67].

Currently, the most extensive experience has been obtained with the treatment of GAMT-deficient patients. The aim of the treatment is to increase the creatine and decrease the guanidinoacetic acid in brain. The severe phenotype observed in some cases in GAMT-deficient patients might be due to both the lack of creatine, but also due to guanidinoacetic acid accumulation, since guanidino compounds are known for their neurotoxic and epileptogenic effects [50]. In order to lower guanidinoacetic acid levels, AGAT activity needs to be reduced. This can be partly achieved by two different strategies:

- Substrate deprivation of AGAT by dietary restriction of arginine intake;
- With supplementation of high concentrations of ornithine and creatine.

With this therapeutic approach, levels of guanidinoacetic acid are lowered by 50% in bodily fluids [51]. This led to an additional clinical improvement as compared with treatment with creatine alone.

No successful treatment has currently been reported for SLC6A8 deficiency. Attempts with creatine supplementation in males showed no marked improvement [25,28,52,53]. Current trials are aiming at stimulation of creatine biosynthesis in the brain by supplementation with high doses of arginine and glycine, combined with high doses of creatine (Mancini GM, van der Knaap MS, Salomons GS, Unpublished Data). However, despite the expression of biosynthesis proteins in the brain, the spatial distribution of AGAT, GAMT and SLC6A8 may pose problems for this approach [54,55].

Creatine: a novel neuromodulator?

Whilst the function of creatine in energy metabolism has been addressed extensively, only a limited number of studies have focused on its role in the brain. Creatine synthesis has been observed in the CNS [56]. *In situ* hybridization studies also found *AGAT* and *GAMT* expression in almost all CNS cell types, in addition to its expression at the blood–brain barrier level, whereas SLC6A8 mRNA was only found in neurons, oligodendrocytes and brain capillary endothelial cells [54,55,57]. These data support the recent postulations that the brain is able to synthesize creatine.

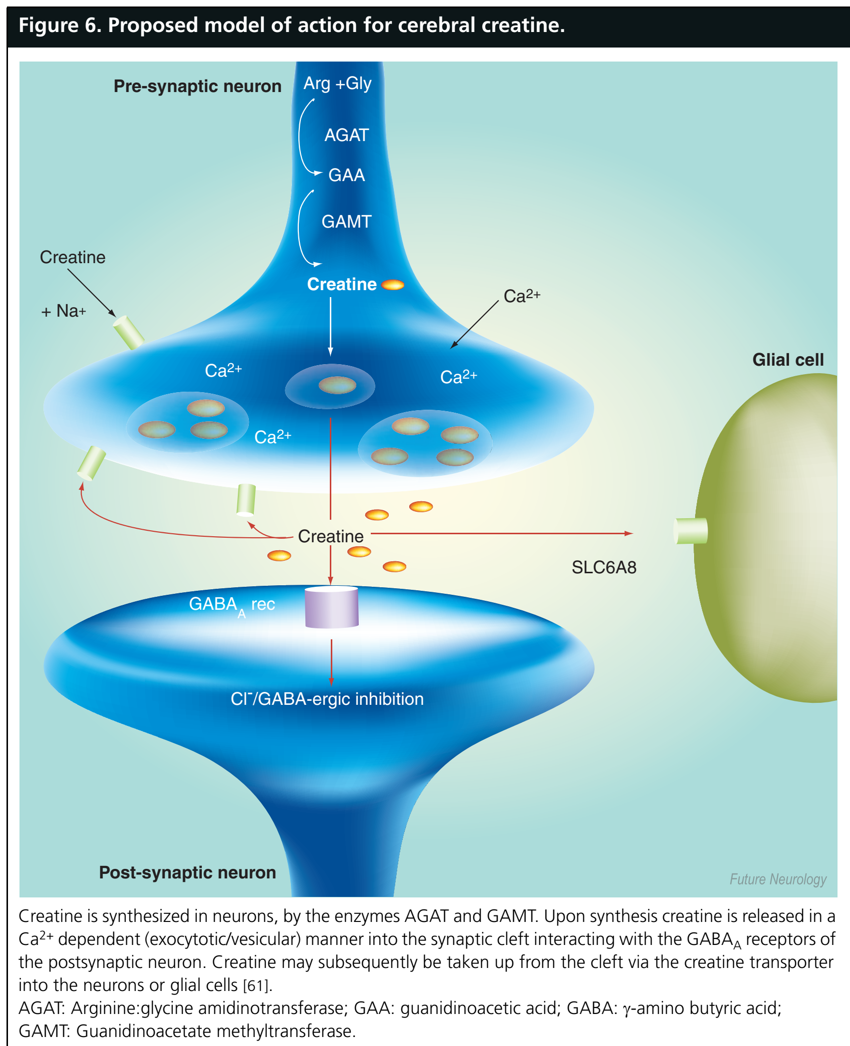
Creatine seems to be essential for brain function, as patients suffering from a CCDS have mental retardation, speech and language disorders, autistic features and may have extrapyramidal movement disorders and epileptic seizures (see above). Additionally, several guanidino

compounds are neurotoxic, presumably by altering the functioning of inhibitory and excitatory amino-acid receptors [58]. Considering the effects of creatine on central neurotransmission processes, it has been shown that guanidino compounds, including creatine, may affect γ-aminobutyric acid (GABA)-ergic neurotransmission as (partial) agonists for $GABA_A$ receptors [58–60]. Indeed, it was shown that creatine is released from central neurons in a manner similar to that of classical neurotransmitters, specifically, involving an exocytotic release mechanism [61]. In view of this *in vitro* data, it was hypothesized that creatine represents a cotransmitter in the brain that modulates the functioning of postsynaptic receptors for neurotransmitters, such as GABA (**Figure 6**) [61].

Conclusions

In the last decade, a novel group of inborn errors of metabolism has been identified: the cerebral creatine deficiency syndromes. The importance of considering CCDS in the differential diagnosis of mental retardation is emphasized by the relative high frequency of SLC6A8 deficiency, and the promising results of treatment of GAMT- and AGAT-deficient patients. The diagnostic tests to detect these syndromes are readily

Figure 6. Proposed model of action for cerebral creatine.



Creatine is synthesized in neurons, by the enzymes AGAT and GAMT. Upon synthesis creatine is released in a Ca^{2+} dependent (exocytotic/vesicular) manner into the synaptic cleft interacting with the $GABA_A$ receptors of the postsynaptic neuron. Creatine may subsequently be taken up from the cleft via the creatine transporter into the neurons or glial cells [61].
AGAT: Arginine:glycine amidinotransferase; GAA: guanidinoacetic acid; GABA: γ-amino butyric acid; GAMT: Guanidinoacetate methyltransferase.

available (e.g., metabolite and molecular analysis and, to a lesser extent, proton MRS), which makes this feasible for any institution that has access to at least one of these techniques. Moreover, proton MRS/MRI in particular enables the detection of multiple diseases. The recent findings that creatine acts as a neuromodulator opens up new research areas, which may be worthwhile for the elucidation of the pathophysiology of CCDS.

Future perspectives

Within the next 5–10 years in the Western world, every mentally retarded patient (male/female) will be screened for CCDS. This could be achieved primarily by proton MRS, which will certainly become more widely available, with the advantage of disclosing additional diseases. For the screening of CCDS, proton MRS is adequate, as a marked reduction or absence of the creatine signal is diagnostic for primary creatine deficiency. Currently, molecular analysis is performed by direct DNA sequencing. However, current developments in the field of microarrays allow the analysis of multiple genes, or even full genomes, in a single assay, which opens up a new world in both research and clinical applications. This technology may lead to more insights into the pathophysiology. The development of sequencing chips (i.e., resequencing arrays) also discloses new perspectives in the diagnosis and screening of CCDS, although its sensitivity is not yet acceptable for clinical diagnostics.

Treatment of SLC6A8 deficiency is one of the big challenges. Clinical improvement has been observed in the patients with biosynthesis defects upon treatment, with almost complete restoration of creatine in the brain. This proves that cerebral creatine restoration is essential. When a vehicle for creatine uptake in the brain is found, treatment should also be successful for SLC6A8 deficiency. Elucidation of the creatine biosynthesis pathway in the brain, in addition to the clarification of the function of creatine in the brain, may increase the success rate of treatment.

Tandem mass spectrometry has the potential for simultaneous multidisease screening and has recently been applied to neonatal screening programs. In case measurement of guanidinoacetic acid in dried blood spots proves to be specific and sensitive enough for detection/exclusion of GAMT deficiency, this disorder will be included in neonatal screening [32,33,13]. AGAT and SLC6A8 deficiencies are not yet eligible for neonatal screening since creatine and creatinine do not seem to be informative in the neonatal period.

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Executive summary

Inborn errors of proteins involved in creatine biosynthesis & its transporter

- Three novel disorders in creatine metabolism have been identified. Two autosomal recessive creatine biosynthesis defects (*AGAT* and *GAMT* deficiency) and one X-linked creatine transporter defect (*SLC6A8* deficiency). This group is defined as cerebral creatine deficiency syndromes (CCDS).
- The common hallmarks of CCDS are the absence, or marked reduction of the creatine signal in the proton magnetic resonance spectroscopy (MRS) of the brain, mental retardation and speech and language delay (Table 1).
- Worldwide, 29 patients with *GAMT* deficiency, five with *AGAT* deficiency and over 34 with *SLC6A8* deficiency have been reported.

High prevalence of cerebral creatine deficiency syndromes in the mental retardation population

- The prevalence of *SLC6A8* deficiency in patients with mental retardation is relatively high (~1%) compared with most monogenic causes of mental retardation.
- The prevalence of the autosomal recessive disorders AGAT- and GAMT-deficiency, is not high, however, a high carrier rate of a *GAMT* (founder) mutation exists in Mediterranean countries.
- The relatively high prevalence of CCDS highlights the importance of including them in the differential diagnosis of mental retardation of unknown etiology.

How to diagnose cerebral creatine deficiency syndromes?

- CCDS may be found either by proton MRS, metabolite screening and/or molecular investigations.
- Biochemically, AGAT-deficiency is characterized by low creatine and guanidinoacetic acid levels in bodily fluids, whereas GAMT-deficiency is characterized by elevated levels of guanidinoacetic acid in bodily fluids.
- Elevated urinary creatine:creatinine ratios are found in males affected with SLC6A8 deficiency.
- In females, the urinary creatine:creatinine ratio is not usually informative in this X-linked disorder, thus mutation analysis of *SLC6A8* is currently the primary choice.
- Prenatal diagnosis of CCDS is possible in families with affected individuals.

Executive summary

Diagnostic pitfalls

- AGAT deficiency is likely under-diagnosed because the biochemical tests may not be sensitive or specific enough to detect decreased metabolite levels.
- In approximately 10% of males with mental retardation in whom elevated urinary creatine:creatinine is found, no *SLC6A8* mutation is detected.
- False-negative biochemical findings (i.e., normal urinary creatine:creatinine ratio) are found frequently in females with heterozygous *SLC6A8* mutations.
- In molecular diagnostic screening, proper criteria should be used to classify novel sequence variants in the CCDS genes as pathogenic mutations.

Treatment of creatine biosynthesis defects & creatine transporter defect

- Treatment approaches of CCDS aim to restore cerebral creatine levels.
- The successful treatment of creatine biosynthesis defects consists primarily of daily creatine supplementation, which preferably should be initiated early in life. In addition, in GAMT deficiency, guanidinoacetic acid levels can be reduced by arginine restriction and ornithine supplementation.
- Current trials for the treatment of SLC6A8 deficiency aim at the stimulation of cerebral creatine biosynthesis by supplementation with high dosage of the substrates of the AGAT enzyme (i.e., arginine and glycine).

Creatine: a novel neuromodulator?

- Cerebral creatine biosynthesis has been proven by the demonstration of *AGAT* and *GAMT* mRNA expression in the brain.
- *In vitro* data indicate that creatine represents a novel cotransmitter in the brain.

Conclusions

- Cerebral creatine deficiency syndromes are, in part, treatable disorders.
- Diagnostic tests for the detection of cerebral creatine deficiency syndromes are widely available.
- Cerebral creatine deficiency syndromes should be included in the differential diagnosis of mental retardation, owing to their high frequency.

Future perspectives

- In the Western world, every mentally retarded patient (male/female) will be screened for CCDS, primarily by proton MRS.
- Novel techniques will increase the number of patients detected with CCDS.
- By identifying a vehicle for creatine uptake in the brain, treatment will also become available for patients with SLC6A8 deficiency.
- GAMT deficiency will be included in neonatal screening programs as guanidinoacetic acid seems to provide a suitable marker, as measured in blood spots by tandem mass spectroscopy.

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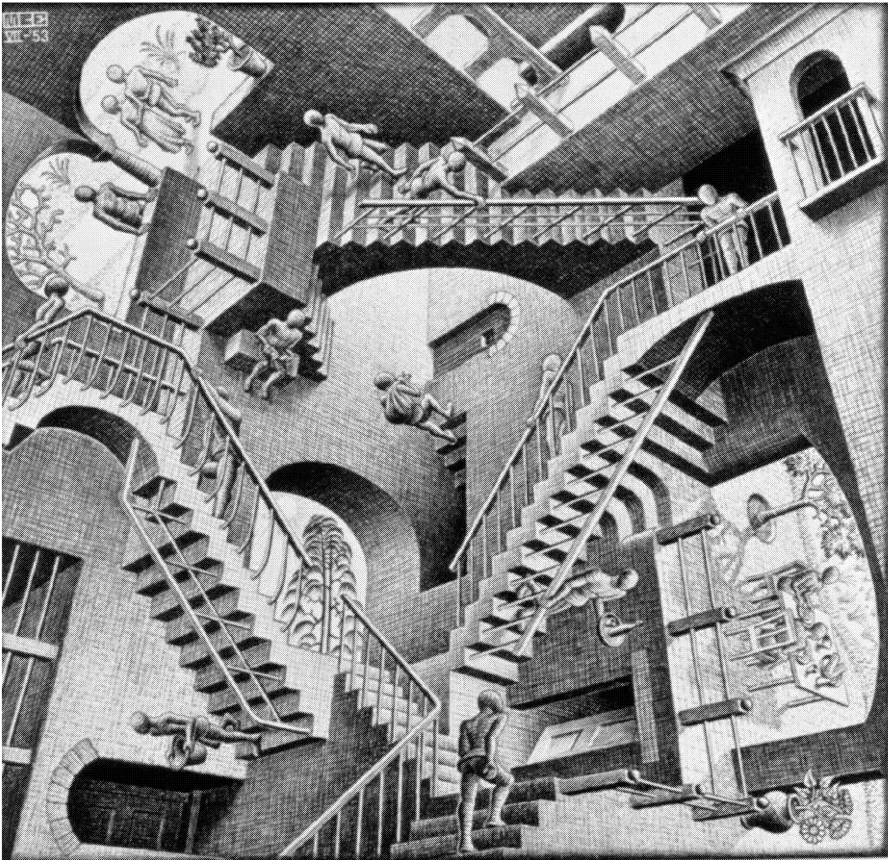
Chapter 8

Summary/discussion/perspectives



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Summary & discussion

The first patient affected with SLC6A8 deficiency was described in 2001 [1]. The diagnosis of mild developmental delay with central hypotonia was initially made at 7 months of age. Examination at 6 years of age showed a severe delay in speech and language development. Magnetic resonance imaging (MRI) and proton magnetic resonance spectroscopy (MRS) of the brain were performed because of progressive increase of the head circumference. Although this was essential in recognizing this novel disorder, macrocephaly is not a clinical sign of the disorder. On proton MRS of the brain, an almost complete loss of the creatine and phosphocreatine signal was observed. The patient did not benefit from oral creatine supplementation, since neither the cerebral creatine concentration on follow-up proton MRS examination, nor clinical symptoms improved. Therefore, oral creatine supplementation was discontinued. The mother and grandmother of the patient had a history of severe learning disability and the maternal uncle of the patient was diagnosed with severe mental retardation [2]. The clinical presentation of a mild phenotype in females vs. severely affected males suggested an X-linked pattern of inheritance. Laboratory workup showed an increased urinary creatine/creatinine ratio in combination with normal urinary guanidinoacetic acid, which ruled out a creatine biosynthesis defect in this patient. The decreased creatine signal on proton MRS, which could not be restored by oral creatine supplementation, in contrast to the high urinary creatine/creatinine ratio suggested a defect in cellular creatine transport. The inheritance pattern suggestive for X-linked disease and the fact that the gene encoding the creatine transporter, *SLC6A8*, is mapped to Xq28 [3] strengthened this hypothesis. Indeed, sequence analysis of *SLC6A8* revealed a hemizygous nonsense mutation p.Arg514X; c.1539C>T [4]. The carrier status for this mutation was confirmed in the female relatives of the patient. Furthermore, it was shown that creatine uptake was impaired in primary fibroblasts of the patient. Hereto, fibroblasts were incubated with creatine, and subsequently the intracellular creatine was measured by gas chromatography mass spectrometry (GCMS) with stable isotope labeled creatine as internal standard. Final proof was provided by restoration of the creatine uptake profile in SLC6A8-deficient fibroblasts after stable transfection with the SLC6A8 wild-type coding sequence (chapter 2 of this thesis).

Within the first years after the recognition of SLC6A8 deficiency, more unrelated families affected with SLC6A8 deficiency, including several within one metropolitan area, were identified, with mental retardation as clinical hallmark. This raised the hypothesis that *SLC6A8* mutations are a relatively frequent cause of mental retardation. Therefore, a collaboration with the European X-linked Mental Retardation (XLMR) Consortium (EurMRX) was initiated. DNA sequence analysis in 288 unrelated male patients with mental retardation of presumed X-linked origin resulted in the detection of 6 pathogenic mutations (p.Gly87Arg, p.Phe107del, p.Tyr317X, p.Cys337Trp, p.Pro390Leu, p.Pro554Leu), demonstrating a prevalence of 2.1% (CI: 0.44%-3.8%) in that panel (Chapter 3).

Considering the estimation that only 10% of the patients with mental retardation are affected with an X-linked defect, the prevalence of 2.1% in XLMR would translate to approximately 0.2% in the general MR population of unknown etiology. This was investigated in a panel of 478 unrelated male patients with idiopathic mental retardation, receiving services through the South Carolina Department of Disabilities and Special Needs (DDSN). Four pathogenic *SLC6A8* mutations were found (p.Asn336del, c.1016+2T>C, Ile347Ile, Arg391Trp), resulting in a prevalence of 0.8% (CI: 0.02-1.7%) (Chapter 4). It should be noted that about half of the patients referred to the South Carolina DDSN have mental retardation of unknown etiology. Therefore, the incidence of SLC6A8 deficiency in the general population of patients with mental retardation is estimated to be close to 0.5%. The relative high prevalence in the mental retardation panel of unknown etiology compared to the XLMR panel may be explained by the selection in the latter panel for possible X-linked inheritance, thus excluding patients with a *de novo SLC6A8* mutation. Indeed, in diagnostic settings *de novo* mutations are found, but not to the extend that this explains the difference. Conversely, the inclusion criteria for the XLMR panel, i.e. at least two male patients within one family, may result in the inclusion of families in which autosomal inheritance is masked by a small pedigree. In the mental retardation panel, described in chapter 4 of this thesis, no indication was found for X-linked inheritance.

A recent retrospective study in 92 males with developmental delay, referred for brain MRS during the time course of one year, revealed 2 cases of creatine transporter deficiency [5]. This study corroborates the findings of the relatively high incidence of SLC6A8 deficiency. However, all studies hamper from a limited number of tested patients. Within the next five to ten years, when more diagnostic laboratories will be screening for cerebral creatine deficiency syndromes (CCDS), the incidence will become more clear.

At present, the majority of *SLC6A8* mutations are unique for the families in which they are found. Mutation types include insertions, single amino acid deletions, large deletions, nonsense mutations, splice mutations and missense variants. Most of these sequence variants clearly cause a change or truncation of the amino acid sequence and are thus classified pathogenic. In case a new variant is detected additional research is needed (e.g. urinary creatine/creatinine ratio, proton MRS of the brain), which should substantiate the diagnosis of SLC6A8 deficiency. If no such material is available, variants can be classified on theoretical grounds: cosegregation of the variant within the family, conservation of the affected amino acid, difference in chemical properties of the substituted amino acid and absence of the variant in at least 210 male control chromosomes [6]. However, missense variants remain particularly difficult to classify. Therefore, we developed an overexpression assay to investigate *in vitro* the nature of such variants (chapter 5). The use of an expression vector with the SLC6A8 coding sequence, allowed us to study the effect of *SLC6A8* variants in primary SLC6A8-deficient fibroblasts. Thus, we were able to classify three potential pathogenic missense substitutions, found in the European XLMR and American mental retardation panels, as rare non-pathogenic variants (p.Lys4Arg, p.GlyG26Arg, p.Val629Ile), and to prove the pathogenic nature of nine other

variants (p.Gly87Arg, p.Phe107del, p.Tyr317Trp, p.Asn336del, p.Cys337Trp, p.Ile347del, p.Pro390Leu, p.Arg391Trp, p.Pro554Leu). For analysis of novel intronic sequence variants (IVS) or translational silent variants, RNA analysis by reverse transcriptase PCR may reveal aberrant splicing of the pre-mRNA. However, if RNA is not available, the pathogenic effect of an IVS can be studied by introducing a genomic fragment containing adjacent exons and or IVS's in a minigene [7]. Subsequently, the construct can be expressed *in vitro*, and the resulting RNA can be examined for alternative splicing. This technique is laborious and time consuming, therefore we suggest to use splice predictor software and/or exonic-splice-enhancer (ESE) predictors (http://www.creatinedeficiency.info/webtools) to check if aberrant splicing is predicted, and only if this is the case, to continue with the above suggested cloning procedure. However, for diagnostic screening of SLC6A8 deficiency and the other CCDS we recommend to first perform the available laboratory tests. The order and need of each test depends on the information that already has been collected. For this we propose a detailed scheme in chapter 5 of this thesis. In some cases this is either not available or not sufficient for a definitive diagnosis. Then, conclusive results on the pathogenic nature of an exonic or IVS variant can be obtained by applying overexpression studies followed by a functional assay (chapter 5) and or mRNA analysis.

In the future, the growing number of recognized patients affected with SLC6A8 deficiency will allow a definition of the clinical presentation and eventually elucidate if a phenotype/genotype relationship exists. The characteristics and the clinical phenotype of patients, identified in the screening of the cohort of chapter 3, as well as patients described in the literature are summarized in chapter 6 of this thesis. One important finding is that SLC6A8 deficiency is a slowly progressive disease. Therefore, if treatment will prevent deterioration of the patients condition, this is a great achievement. Ideally, brain creatine should be restored and a rise in IQ and speech and language development will be reached.

In chapter 7 an overview is provided on the discovery, diagnosis and clinical characteristics of the CCDS, with a focus on the future developments in CCDS. As discussed above, the use of proton MRS has resulted in the identification of three inborn errors of metabolism in creatine biosynthesis (AGAT deficiency, MIM 602360; GAMT deficiency, MIM 601240) or transport (SLC6A8 deficiency, MIM 300036) [8-14], [1,4,15-18]. The biosynthesis defects, AGAT and GAMT deficiency, are autosomal recessive disorders, while the transporter defect, SLC6A8 deficiency is X-linked, as mentioned above. The first metabolic disorder of creatine metabolism, GAMT deficiency was recognized in 1994 [10]. The second and third inherited disorders of creatine metabolism were both described in 2001: deficiency of the creatine transporter [4] and AGAT deficiency [9]. Characteristic in the clinical presentation of all CCDS are mental retardation, expressive speech and language delay and epilepsy (varying from intractable seizures in GAMT deficient patients to mild epileptic or febrile seizures in AGAT deficient and transporter deficient patients). GAMT deficient patients and transporter deficient patients may show autistic like behavior [18,19]. Metabolite measurements in urine and plasma are

indicative for the specific disorders; e.g. in SLC6A8 deficient males the creatine/creatinine ratio is increased in urine, and guanidinoacetate is increased in urine and plasma of GAMT deficient patients, whereas guanidinoacetate is decreased in plasma of AGAT deficient patients [20]. The CCDS share the almost complete lack of creatine/phosphocreatine in the brain measured by in vivo proton MRS.

In patients suffering from a CCDS the brain function is most affected, and thus creatine must be essential for brain function. Recently, our group showed in collaboration with the group of Prof. Schoffelmeer, that in rat brain slices, creatine is released from central neurons in a manner similar to that of classical neurotransmitters, i.e. involving an exocytotic release mechanism [21], strongly suggesting a brain specific role of creatine.

Indeed, recent studies showed that the brain is able to synthesize creatine, since AGAT and GAMT are expressed by every CNS main cell type (neurons, astrocytes and oligodendrocytes). SLC6A8 was only found in neurons and oligodendrocytes and brain capillary endothelial cells [22,23]. There is evidence that these three genes are usually not expressed within the same cell. This could pose problems for treatment stategies in SLC6A8 deficiency as discussed below, in which the cerebral creatine synthesis is the target of treatment. Guanidinoacetic acid, the product of AGAT, needs to be taken-up by the neighbouring cell via SLC6A8 in order to be methylated by GAMT, thus forming creatine.

Treatment approaches of CCDS aim to restore creatine in the brain by creatine supplementation with favorable results for the biosynthesis defects. For AGAT deficiency the highest success rate is expected because there is no accumulation of (neurotoxic) substrates. In fact, creatine supplementation in AGAT and GAMT deficient patients leads to an impressive restoration of cerebral creatine levels, and a favorable clinical response if treated at young age [24,25]. This suggests that creatine supplementation early in life prevents the neurological sequelae. To date, no successful treatment has been reported for SLC6A8 deficiency. Attempts with creatine supplementation only, in males, showed no marked improvement [1,4,16,26]. Current trials are aiming at stimulation of creatine biosynthesis in brain by supplementation with high doses of arginine and glycine, combined with high doses of creatine (unpublished data from drs GM Mancini, MS van der Knaap and GS Salomons), which may hamper from the spatial distribution of AGAT and GAMT. When a vehicle for intracellular creatine delivery in brain will be found, treatment should also be successful in SLC6A8 deficiency. Clarification of the function of creatine in brain may eventually increase the success rate of treatment.

Mental retardation can result from environmental causes (e.g. trauma, infections, perinatal asphyxia), chromosomal aberrations (e.g. rearrangements, trisomy) and genetic causes (e.g. gene mutations). In case patients can be grouped according to similar symptoms, they display syndromic or symptomatic mental retardation. Often, no clear symptoms besides mental retardation can be assigned to the patients, therefore these patients suffer from non-syndromic or non-symptomatic mental retardation. Occasionally, mutations in the same gene (e.g. *MeCP2*) may result in syndromic mental retardation in some cases, and non-syndromic mental retardation in other cases [27-30], thus making

proper diagnosis difficult [31,32]. Furthermore, if tests are not performed (e.g. brain proton MRS) or parameters are not tested (e.g. plasma creatine and guanidinoacetic acid, urinary creatine/creatinine ratio and guanidinoacetic acid), diagnosis may be missed in certain cases. Therefore, extensive examination of patients, including broad laboratory testing, is not only important for the examined patient, but may reveal newly recognized, perhaps frequent, disorders. This was particularly true for SLC6A8 deficiency, in which brain proton MRS lead to the recognition of the first patient with SLC6A8 deficiency. XLMR genes have enjoyed great attention over the last years, however, autosomal monogenic causes of MR should not be neglected [33]. In conclusion, the relatively large contribution of the CCDS to the monogenic causes of mental retardation, emphasizes the importance of including CCDS in the differential diagnosis of mental retardation of unknown etiology and the need of broad unprecedented examination of idiopathic mental retardation.

Future perspectives

The growing awareness of CCDS as a frequent cause of mental retardation, will result in a higher percentage of accurate diagnoses of mentally retarded patients. For detection of CCDS, proton MRS is important, as a marked reduction or absence of the creatine signal is diagnostic for primary creatine deficiency. Furthermore, proton MRS has the advantage of the possibility of disclosing additional diseases. Nevertheless, this technique is not yet commonly available, which likely will improve within the near future. Currently, molecular analysis is performed by direct DNA sequencing. However, current developments in the field of microarrays allow the analysis of multiple genes or even full genomes in a single assay, which opens a new world in both research and clinical applications. The development of sequencing chips (i.e. resequencing arrays) discloses new perspectives also in the diagnosis of CCDS, although its sensitivity is currently not yet acceptable for the clinical diagnostics.

The number of diseases that are tested in the Dutch neonatal screening in bloodspots by tandem MS will soon be been increased to 18 (http://www.minvws.nl/). Important criteria for the selected diseases were high sensitivity and specificity of the test and treatability of the disease. The latter is nearly guaranteed in GAMT and AGAT deficiencies, especially if treated in the neonatal period [24,25]. Moreover, sufficient sensitivity and specificity can be achieved for elevated guanidinoacetic acid measurement on tandem MS in case of GAMT deficiency. In Mediterranean countries, neonatal screening for GAMT deficiency may be especially warranted, as GAMT deficiency appears to be more frequent in that region than in other (Western) countries [34,35]. Inclusion of SLC6A8 deficiency, being the more frequent cause of CCDS, in neonatal screening is currently not reasonable because of 1) the lack of successful treatment, 2) no biochemical marker in bloodspots available, 3) creatine/creatinine ratio in urine is not specific enough and 4) the need of an additional independent

urine test. Moreover, a complicating factor is that this urine test is usually not informative for females with a heterozygous pathogenic *SLC6A8* mutation.

Families at risk for a heritable disease are usually offered prenatal diagnosis by amniocentesis or chorionic villi sampling. Considering the increased risk of spontaneous abortion due to invasive procedures, methods that are non-invasive are welcome. One such method could be fetal cell-free circulating nucleic acids in maternal serum and plasma [36]. Likely, within ten years the ongoing attempts to distinguish cell-free circulating fetal DNA and RNA from the maternal counterparts will result in non-invasive prenatal testing of CCDS. Since SLC6A8 deficiency mainly affect males, families may wish to perform invasive prenatal diagnosis only in case of a male fetus. Then Y-chromosome detection in maternal plasma can be used to select male carrying pregnancies for further testing (i.e. chorionic villi sampling and amniocentesis).

Treatment strategies in SLC6A8 deficiency may benefit from better understanding of the structure/function relationship of the SLC6A8 protein. Mutations that affect protein function but not maturation to the plasma membrane may require a different approach than mutations that cause protein retention in the cytoplasm rather than protein function. In the first case, creatine derivatives may be successful in treatment, wereas in the latter case, pharmacological rescue of the creatine transporter may be feasible, as suggested for the carnitine transporter (SLC22A5) [37]. Although oral creatine supplementation has not resulted in restoration of brain creatine, treatment may result in revelation of other symptoms, such as the gastrointestinal problems that are described in chapter 5, thus improving quality of life of these patients. Furthermore, female carriers of a pathogenic *SLC6A8* mutation may display only a mild phenotype, due to the presence of both a *SLC6A8* allele with and without a mutation and perhaps favourable skewing of X-inactivation, which suggests that treatment strategies to replenish cerebral creatine in female carriers may be easier to accomplish than in male patients.

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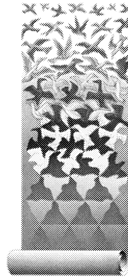
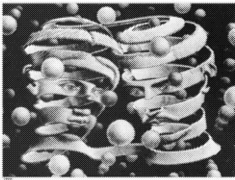
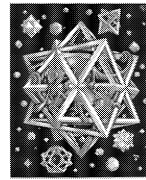
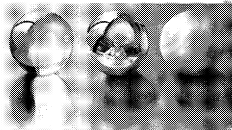
Chapter 9

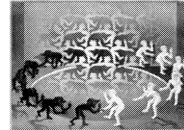
Nederlandse samenvatting

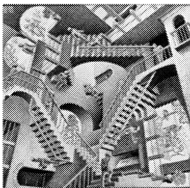


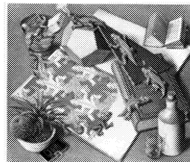












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Nederlandse samenvatting

De eerste patiënt met creatine transporter (SLC6A8) deficiëntie is in 2001 beschreven [1]. In het eerste levensjaar werd bij deze patient ontwikkelingsachterstand vastgesteld en op zes jarige leeftijd werd een vergrote hoofdomtrek geconstateerd. Dit was de reden om proton magnetische resonantie spectroscopie (H-MRS) van het hoofd uit te voeren. Hierbij werd een sterk verlaagd creatine signaal gezien. Behandeling door middel van orale toediening van creatine resulteerde niet in het herstel van het creatine signaal, noch in een verbetering van het klinisch beeld van de patiënt. De familie anamnese toonde ernstige leerproblemen bij de moeder en grootmoeder van de patiënt en een ernstig mentaal geretardeerde (geestelijk gehandicapte) oom van moeders kant [2]. Het milde ziektebeeld bij de vrouwelijke familieleden ten opzichte van de ernstig aangedane mannelijke patiënten suggereerde X-chromosomale overerving. Laboratoriumbevindingen toonden een verhoogde creatine/creatinine verhouding in urine aan, in combinatie met normale guanidinoacetaat concentraties, waarmee een creatine biosynthese defect werd uitgesloten. De feiten: 1) verlaging van creatine in de hersenen 2) orale creatine toediening resulteerde niet in een herstel van de creatine concentratie in de hersenen en 3) verhoogde creatine/creatinine verhouding in de urine, leken te wijzen op een defect in cellulaire creatine transport. Het overervingspatroon passend bij een X-gebonden aandoening en het feit dat het creatine transporter gen op het X-chromosoom ligt [3] versterkten deze hypothese. Inderdaad werd door middel van gen sequentie analyse een nonsense mutatie (p.Arg514X) vastgesteld in deze patiënt [4]. Primaire huid fibroblasten van de patiënt bleken niet in staat creatine te transporteren, hetgeen kon worden aangetoond door de fibroblasten te kweken in aanwezigheid van creatine, gevolgd door het meten van intracellulair creatine door middel van gas chromatografie massa spectrometrie, waarbij stabiele isotoop gelabeld creatine werd gebruikt als interne standaard. Het herstel van de creatine opname in de deficiënte fibroblasten na stabiele transfectie van het coderende gedeelte van het wild-type SLC6A8 gen gaf het definitieve bewijs dat de mutatie in dit gen de primaire oorzaak is van het ziektebeeld.

Binnen enkele jaren werden meerdere niet verwante families met SLC6A8 deficiëntie gediagnosticeerd, waarvan een aantal families binnen één metropool gebied. Dit deed vermoeden dat deze aandoening een relatief frequente oorzaak van mentale retardatie is. Hiertoe werd, in samenwerking met het Europees XLMR consortium, het SLC6A8 gen van 288 patiënten gecontroleerd op veranderingen. Dit resulteerde in de identificatie van zes pathogene mutaties (p.Gly87Arg, p.Phe107del, p.Tyr317X, p.Cys337Trp, p.Pro390Leu, p.Pro554Leu), hetgeen in deze groep een prevalentie opleverde van 2,1% (betrouwbaarheidsinterval: 0.44%-3.8%) (Hoofdstuk 3 van dit proefschrift).

Oorzaken van X-gebonden mentale retardatie worden verondersteld 10% van alle gevallen van mentale retardatie te veroorzaken. Dit zou betekenen dat in slechts 0,2% van de gevallen van idiopathische mentale retardatie sprake is van SLC6A8 deficiëntie. Om dit te onderzoeken werd, in

samenwerking met het Greenwood Genetic Center in South Carolina in de VS, het SLC6A8 gen onderzocht van 488 patiënten met mentale retardatie zonder bekende oorzaak. Hierbij werden vier pathogene *SLC6A8* mutaties gevonden (p.Asn336del, c.1016+2T>C, Ile347Ile, Arg391Trp), hetgeen resulteert in een prevalentie van 0,8% in deze groep (betrouwbaarheids interval: 0,02-1,7%) (hoofdstuk 4 van dit proefschrift). In ongeveer de helft van patiënten, die gezien worden in het Greenwood Genetic Center, wordt geen oorzaak gevonden voor de mentale retardatie, zodat de werkelijk incidentie van SLC6A8 deficientie waarschijnlijk dichter bij de 0,5% ligt. Eén mogelijke verklaring voor de relatief hoge prevalentie in de idiopathische mentale retardatie groep ten opzichte van de XLMR groep is dat voor de laatste groep een selectie is uitgevoerd voor een overervingspatroon suggestief voor een X-gebonden aandoening. Daardoor worden *de novo* gevallen uitgesloten van dit panel. Hoewel in de diagnostiek *de novo SLC6A8* mutaties worden aangetroffen, is het aandeel daarvan in de totale patiënten populatie niet zo hoog dat het dit verschil geheel kan verklaren. Anderzijds leiden de criteria voor inclusie in het XLMR panel, tenminste twee aangedane mannelijke familieleden, ook tot het includeren van kleine families, waardoor autosomale overerving zeker niet is uitgesloten. In het mentale retardatie panel dat is bestudeerd in hoofdstuk 4, werd geen indicatie gevonden voor X-gebonden overerving. Daarentegen was bij de patiënten waarbij een mutatie werd aangetoond de informatie over de families gering en dus kon dit argument niet worden getoetst.

Een recente retrospectieve studie in 92 mannelijke patiënten, die wegens ontwikkelingsachterstand werden verwezen voor H-MRS van de hersenen, bracht twee patiënten aan het licht, waarmee de resultaten van deze studie zich scharen in de zelfde orde van grootte voor SLC6A8 mutatie frequentie (0.5%-2.1%) [5]. Het beperkte aantal patiënten per studie maakt het moeilijk om een statistisch solide prevalentie te berekenen, maar de groeiende aandacht voor SLC6A8 deficiëntie zal de komende jaren leiden tot een betere schatting van de werkelijke incidentie.

De meeste mutaties die tot nog toe gevonden zijn, zijn uniek voor de familie waarin zij zijn aangetroffen. Het spectrum aan DNA varianten bevat kleine en grote mutaties, nonsense- en splice mutaties en missense varianten. De meeste mutatietypen hebben een duidelijk pathogeen karakter, maar wanneer een mutatie voor de eerste keer wordt aangetroffen, moet de diagnose SLC6A8 deficiëntie worden gestaafd met aanvullend patiëntenmateriaal, zoals urine. Wanneer dergelijk materiaal niet verkregen kan worden, zal de variant worden geclassificeerd op theoretische argumenten, zoals overerving binnen de familie, geconserveerdheid van het betreffende aminozuur, chemische verschillen met het oorspronkelijke aminozuur en de afwezigheid van de variant in tenminste 210 mannelijke controle chromosomen [6]. Evenwel zijn vooral de missense varianten moeilijk te classificeren. Hiertoe hebben we een overexpressie methode ontwikkeld om *in vitro* de aard van een dergelijke variant te kunnen beoordelen. Het gebruik van de coderende sequentie van het *SLC6A8* gen in een expressie vector (pEGFP) maakt het mogelijk het effect van deze varianten te testen in primaire SLC6A8 deficiënte fibroblasten. In hoofdstuk 5 van dit proefschrift wordt deze

methode toegepast, waardoor drie varianten als niet pathogeen geclassificeerd werden (p.Lys4Arg, p.Gly26Arg en Val629Ile), terwijl van negen andere varianten kon worden aangetoond dat deze pathogeen zijn (p.Gly87Arg, p.Phe107del, p.Tyr317Trp, p.Asn336del, p.Cys337Trp, p.Ile347del, p.Pro390Leu, p.Arg391Trp, p.Pro554Leu). Voor de analyse van intronische sequentie varianten (IVS) en exonische varianten die geen consequentie hebben voor het betreffende aminozuur (translational silent variants), kan met behulp van reverse transcriptase PCR in RNA onderzocht worden of deze varianten leiden tot aberrante splicing. Wanneer RNA echter niet beschikbaar is, kan dit onderzoek *in vitro* met behulp van een mini-gen worden uitgevoerd [7]. Omdat deze methode arbeidsintensief is adviseren wij eerst te onderzoeken met behulp van voorspellende programma's of er foutieve splicing verwacht kan worden (http://www.creatinedeficiency.info/webtools). Afhankelijk van de beschikbaarheid en uitkomst van diagnostische laboratoriumtestresultaten kunnen verschillende strategieën worden gevolgd om tot een definitieve diagnose te komen. Hiertoe hebben we een gedetaileerd beslisdiagram opgesteld.

Het toenemend aantal patiënten met SLC6A8 deficiëntie zal het in de toekomst mogelijk maken om tot een betere karakterisering van het ziektebeeld te komen. In hoofdstuk 6 van dit proefschrift word een eerste inventarisatie gemaakt van het ziektebeeld van patiënten uit de XLMR groep van hoofdstuk 3 alsmede patiënten die elders in de literatuur beschreven zijn. Een belangrijke bevinding is dat deze aandoening progressief lijkt te zijn. De verwachting is dat behandeling in de toekomst niet zal leiden tot complete genezing. Toch zal het belangrijk zijn minstens de voortschrijdende achteruitgang van de patiënten tot staan te brengen. Idealiter zal de behandeling resulteren in het herstel van het creatine niveau in de hersenen en een verbetering van het IQ en spraak en taal ontwikkeling.

Met behulp van H-MRS zijn in totaal drie aangeboren aandoeningen van de creatine synthese en transport geïdentificeerd: AGAT-, GAMT- en SLC6A8-deficiëntie [8-14], [1,4,15-18]. AGAT- en GAMT-deficiëntie zijn autosomaal recessieve aandoeningen van de creatine synthese enzymen, terwijl SLC6A8-deficiëntie een X-gebonden aandoening is, zoals hierboven vermeld. Als eerste van deze drie cerebrale creatine deficiëntie syndromen (CCDS) werd in 1994 GAMT-deficiëntie opgehelderd [10]. In 2001 volgden SLC6A8- en AGAT-deficiëntie [4,9]. De CCDS leiden alle drie tot een sterke vermindering van creatine in de hersenen, mentale retardatie, achterstand in spraak en taal ontwikkeling en verschillende vormen van epilepsie. Autistisch gedrag kan aangetroffen worden in GAMT- en creatine transporter deficiënte patiënten [18,19]. Hoe het onderscheid tussen de drie CCDS gemaakt kan worden door middel van laboratoriumtests is samengevat in hoofdstuk 7 van dit proefschrift.

Het feit dat in CCDS voornamelijk hersen-gerelateerde symptomen worden aangetroffen, duidt op een essentiële rol voor creatine in de hersenen. Recent onderzoek heeft aangetoond dat de afgifte van creatine in de hersenen vergelijkbaar is met die van klassieke neurotransmitters [20].

Recente studies hebben AGAT en GAMT expressie aangetoond in neuronen, astrocyten en oligodendrocyten in de hersenen, hetgeen duidt op de mogelijkheid van creatine synthese in de hersenen. SLC6A8 is alleen aangetoond in neuronen, oligodendrocyten en hersencapillair-endotheel cellen [21,22]. Deze studies laten zien dat de drie genen over het algemeen niet tegelijk in dezelfde cel tot expressie komen. De compartimentalisatie van de creatine synthese stappen in de hersenen en de creatine transporter is mogelijk een barrière voor succesvolle behandeling van SLC6A8 deficiënte patiënten, die momenteel voornamelijk gericht is op het stimuleren van de biosynthese van creatine in de hersenen. Orale toediening van creatine bij AGAT- en GAMT-deficiëntie, heeft geleid tot enorme klinische verbetering van de patiënten, waarbij vroege interventie leidt tot betere resultaten [23,24]. Opheldering van de rol van creatine in de hersenen zal mogelijk leiden tot succesvolle behandeling van creatine transporter patiënten.

Er zijn talloze oorzaken van mentale retardatie (trauma, infectie, chromosomale afwijkingen, genmutaties). In sommige gevallen zijn er aanvullende symptomen die kunnen helpen bij het vaststellen van de onderliggende oorzaak: symptomatische of syndromale mentale retardatie. In andere gevallen worden geen andere symptomen gevonden, zodat de algemene diagnose asymptomatische of asyndromale mentale retardatie wordt vastgesteld [25,26]. Verschillende mutaties in specifieke genen kunnen leiden tot beide vormen van mentale retardatie in onafhankelijke families. Daarnaast is het belangrijk om te beseffen dat in bepaalde gevallen een specifieke diagnose gemist kan worden door het niet uitvoeren van bepaald onderzoek. SLC6A8 deficiëntie is daarvan een duidelijk voorbeeld: hoewel creatine meting in de urine een toegankelijke test is, wordt dit nog niet standaard aangevraagd voor patiënten met idiopathische mentale retardatie. Niet alleen voor de individuele patiënt is het belangrijk om uitvoerig onderzoek te doen naar de oorzaak van de mentale retardatie, maar het zou ook kunnen leiden tot het ophelderen van een nieuwe oorzaak van asyndromale mentale retardatie. Illustratief hiervoor is het feit dat SLC6A8 deficiëntie is geïdentificeerd naar aanleiding van een vergrote hoofdomtrek, terwijl dat geen algemeen symptoom blijkt te zijn van deze ziekte. Selectie voor X-gebonden overerving heeft flink bijgedragen aan de identificatie van genen die betrokken zijn bij het ontstaan van mentale retardatie, maar ook autosomale gendefecten kunnen daar aan ten grondslag liggen [27]. In mediterrane landen bijvoorbeeld is een hoge dragerschap frequentie aangetoond voor pathogene GAMT mutaties. Bovendien is de meerderheid van de patiënten met GAMT deficiëntie daaruit afkomstig [28,29]. De relatief hoge bijdrage van CCDS aan de gevallen van mentale retardatie van onbekende oorzaak benadrukken het belang van het includeren van deze aandoeningen in de differentiaal diagnose van mentale retardatie.

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