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ORIGINAL ARTICLE

Treatment by oral creatine, L-arginine and L-glycine in six severely affected patients with creatine transporter defect

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Abstract
Background X-linked cerebral creatine deficiency is caused by the deficiency of the creatine transporter (CTP) encoded by the SLC6A8 gene.
Patients and Methods We report here a series of six patients with severe CTP deficiency, four males and two females; clinical presentations include mild to severe mental retardation (6/6), associated with psychiatric symptoms (5/6: autistic behaviour, chronic hallucinatory psychosis), seizures (2/6) and muscular symptoms (2/4 males). Diagnosis was suspected upon elevated urinary creatine/creatinine (except in one of the female patients) and on a markedly decreased creatine peak on magnetic resonance spectroscopy (MRS). Diagnosis was confirmed by molecular analysis that identified four novel mutations not reported so far, including a mutation found twice in two male patients. All patients were treated successively and according to the same protocol by creatine alone then combined to its precursors, L-glycine and L-arginine for 42 months.
Results and conclusion In our patients, creatine supplementation alone or with its precursors L-glycine and L-arginine showed benefit only in the muscular symptoms of the disease and no improvement in the cognitive and psychiatric manifestations and did not modify brain creatine content on MRS of male and female CTP deficient patients. New treatment strategies are required including creatine

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derivatives transported independently from CTP or using alternative pathways and transporters.

Background

Mental retardation coupled with speech and behaviour disorders are clinical hallmarks of the inherited defects of creatine (Cr) metabolism (MIM # 300036). These rare disorders include two defects of Cr synthesis, i.e. arginine: glycine amidino transferase (AGAT, EC 2.1.4.1) and guanidinoacetate (GAA) methyltransferase (GAMT, EC2.1.1.2) deficiencies (Stockler-Ipsiroglu and Salomons 2006) and one defect of Cr transport (i.e. SLC6A8) deficiency (CTP) (Cecil et al. 2001; Salomons et al. 2001). The first two are autosomal recessive diseases, while the latter is X-linked, and could represent up to 1% of mental retardation of unknown origin in males (Clark et al. 2006). All these conditions share a common pathogenetic mechanism, the depletion of brain Cr, which seems to cause a significant alteration in the developing brain and leads to similar phenotypes characterized, in various combinations and severity, by mental retardation, autistic-like behaviour, speech impairment, seizures and movement disorders (Mercimek-Mahmutoglu & Stockler-Ipsiroglu 2009). Although CTP deficiency affects mainly males, a few females have also been described with epilepsy and/or learning disabilities and mild mental retardation (Mercimek-Mahmutoglu et al. 2010; van de Kamp et al. 2011). A few patients also display myopathic features, especially muscular hypotonia (Anselm et al. 2006; Rogers et al. 2008).

Diagnosis is made upon measurement of Cr and GAA in plasma, urine and CSF or by brain magnetic resonance spectroscopy (MRS) showing absence of creatine in the brain in all defects of creatine metabolism. In AGAT deficiency low creatine and GAA levels are found in plasma, urine and CSF whereas in GAMT deficiency low creatine and high GAA levels are the diagnostic hallmarks. In CTP deficiency creatine and GAA are normal in plasma but elevated in urine in male patients but an inconstant finding in females (Almeida et al. 2004). Enzyme assays and molecular studies can be performed for AGAT and GAMT deficiencies to confirm the diagnosis. Molecular or functional studies of creatine uptake in cultured cells are used to confirm CTP deficiency (Salomons et al. 2001).

Clinical symptoms in AGAT and GAMT deficiencies can be partially reversed by oral Cr supplementation associated with a low arginine (Arg) and glycine (Gly) diet aiming at reducing GAA synthesis and accumulation in GAMT deficiency (Leuzzi et al. 2000). In a few patients early treatment in asymptomatic patients totally prevented the occurrence of clinical manifestations (Battini et al. 2006). Ornithine administration has also been proposed in GAMT deficiency to decrease GAA synthesis, a neurotoxic compound that accumulates in the grey matter by inhibiting AGAT enzyme (Sipilä 1980). In conclusion in AGAT and GAMT defects Cr supplementation leads to clinical improvement and correction of brain creatine depletion as depicted by the normalization of the MRS within a few months of treatment (Mercimek-Mahmutoglu et al. 2006).

On the contrary, until now, no effective therapy has been available for CTP deficiency based on Cr monotherapy (de Grauw et al. 2002; Newmeyer et al. 2005; Anselm et al. 2006) or combined to its precursor L-Arg (Fons et al. 2008) except in two recently reported cases of combined treatment associating Cr and L-Arg (Chilosi et al. 2008) or creatine, L-Arg and L-Gly (Mercimek-Mahmutoglu et al. 2010).

Here we report a series of six patients with severe CTP deficiency, four males and two females, presenting with neurocognitive, psychiatric and myopathic symptoms, treated successively and according the same protocol by Cr alone then combined to its precursors, L-Gly and L-Arg.

Patients

Patients 1 to 3, all males, presented with severe mental retardation, behaviour disturbance, principally autistic behaviour and concentration disability and in two patients febrile and non febrile seizures. Two of the males (patients 1 and 3) also displayed myopathic symptoms with muscular hypotonia and gross motor delay but normal CK levels (Table 1).

Patients 4 and 5, both females, presented with severe mental retardation, autistic spectrum and behaviour disturbance. Patient 4 developed myoclonic and absence epilepsy whereas patient 5 displayed psychiatric manifestations (chronic hallucinatory psychosis, Table 1).

Patient 6 presented with a milder phenotype, mild psychomotor retardation and speech delay but had no behaviour disturbance neither seizures. Patients 1, 3 and 4 received anti-epileptic drugs at treatment onset.

In all but patient 3, the diagnosis of CTP deficiency was suspected in brain MRS before any urinary screening. All males had undetectable Cr peak on brain MRS while the two females showed low Cr peak (Fig. 1). In patient 3, high Cr in urine was found before performing MRS, which also showed absence of creatine (Table 1). In other male patients Cr urinary levels were elevated while GAA urinary and plasma levels and plasma Cr were in the normal range. By contrast in the two female patients urinary Cr was normal in patient 4 and in the upper normal range in patient 5 (Table 1).

The diagnosis was confirmed in all cases by identifying a mutation in the *SLC6A8* gene (Table 1). Four sequence variations (patients 1,3,4,6; Table 1) were not previously

Table 1 Clinical, biochemical, molecular and MRS data of six patients affected with creatine transporter deficiency before and after treatment

Pat.	Sex	Age at DG (ys)	SLC6A8 mutation Urinary creatine Urinary GAA μmol/mmol creatinine (reference range)	Seizures/ cognitive status (test) Before treatment	 After n months of treatment	Behavior disturbances/ psychiatric manifestations Before treatment	 After n months of treatment	Muscular symptoms CK levels (N<180) Before treatment	 After n months of treatment	Brain Creatine (MRS) Before treatment	 After n months of treatment
P1	M	2	c.541T>C; p.(Cys181Arg) uCr: 4027 (6–1208) uGAA: 180 (4–220)	2 y2 m: febrile and non febrile seizures IQ/DQ: not evaluable Speech delay	5 y6 m: no speech failed construction activities and image-object pairing	Autistic behavior	Unchanged	Muscular hypotonia Walked unsupported at 2 years CK: 120 U/L	Increase in muscular mass and strength. Improved gross motor skills	Absent	Absent
P2	M	3	c.1221_1223delTTC; p.(G414del) uCr: 5613 (6–1208) uGAA: 87 (4–220)	3 ys: no seizures IQ/DQ: developmental age of 9 months No speech	6 ys: impossible to evaluate due to hyperactive behavior	Autistic behaviour with hyperactivity and emotional instability	Hyperactivity deteriorated, seeking sensory stimulations	Absent	Absent	Absent	Absent
P3	M	2.5	c.1519_1543del; p.(Ile507LeufsX5) uCr: 3042 (6–1208) uGAA: 120 (4–220)	4 y: Febrile seizures Postural DQ =30 m OM coord. DQ=19 m makes some sounds, points to pictures	7 y: failed to perform IQ tests	Autistic behaviour, seeking sensory stimulations	Hyperactive behaviour deteriorated.	Muscular hypotonia Walked unsupported age 26 months CK: 87 U/L	Improved gross motor functions	Absent	Absent
P4	F	16	c.291_292insAGGG; p.(Ala98ArgfsX92) uCr: 174 (11–244) uGAA: 19 (3–78)	16 y: myoclonus-absence epilepsy IQ =40 (VCI=45 PRI=45 PSI=50, WMI=50)	Epilepsy not improved 19 y: IQ=39	Autistic behaviour	Unchanged	Absent	Absent	Reduced (50% normal)	Unchanged
P5	F	14	90% aberrant spliced SLC6A8 c.263-1G>C; p.(Gly88Leu108del) uCr: 401,515,541 (11–244), uGAA: 58 (44–220)	No seizures 14 y3 m: IQ=45 (WISC IV) VCI=61, PRI=45 WMI=53, PSI=66	17 y 6 m: IQ=48 (WISC IV)	Chronic Hallucinatory psychosis Massive anxiety, Altered contact and adaptability	Reduced frequency of hallucination episodes	Absent	Absent	Reduced (40% normal)	Unchanged
P6	M	5	c.1519_1543del; p.(Ile507LeufsX5) uCr: 2163 (17–721) uGAA: 219 (4–220)	No seizures 5 y 5 m DQ=50 (Brunet-Lézine)	8 y6 m DQ=43 (Brunet-Lézine)	No behavior disturbance	Unchanged	Absent	Absent	Absent	Absent

Abbreviations: m: months, y: years, IQ: intelligence quotient, DG: diagnosis, DQ: development quotient, OM coord = oculomotor coordination, VCI = verbal comprehension index, PRI = perceptual reasoning index, WMI = working memory index, PSI = processing speed index

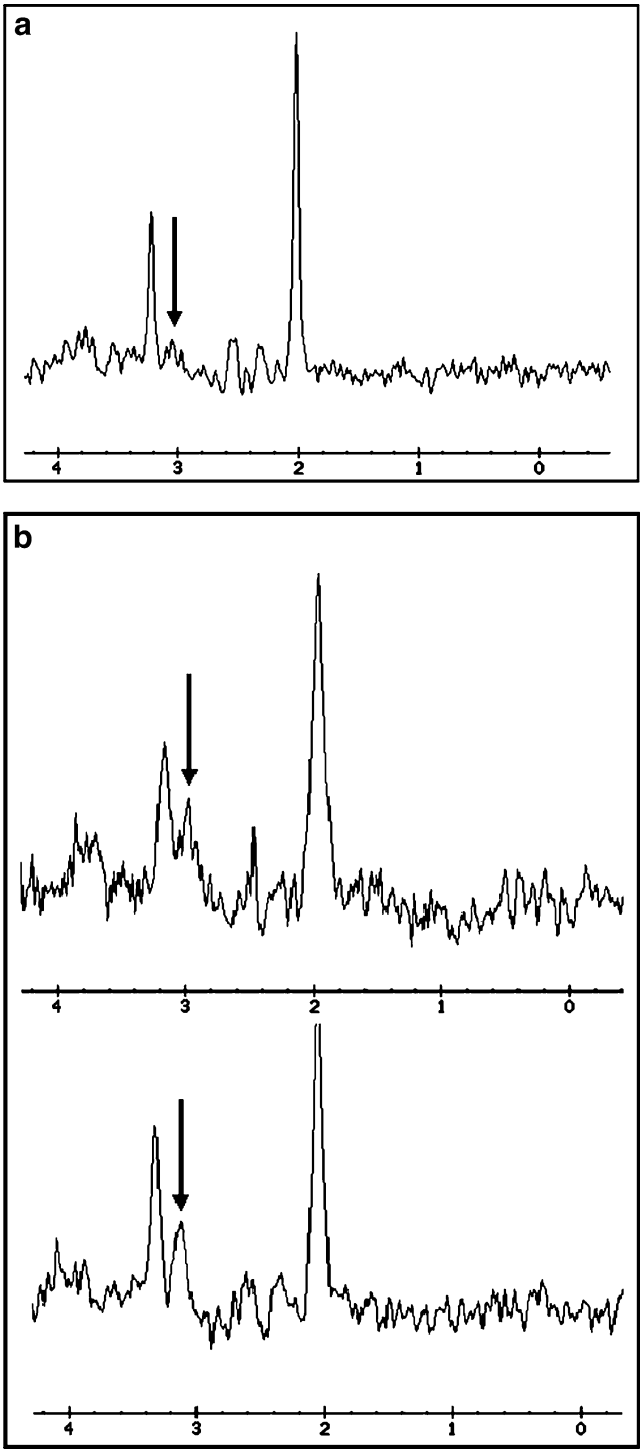

Fig. 1 Brain magnetic resonance spectroscopy (MRS) scan from one of the male patients (**a**, P1) and from the two female patients (**b**, P4 upper frame, P5 lower frame). The black arrows depict the creatine peak position. All other male patients displayed similarly to P1 absent creatine peak on MRS (not shown)

reported at the time of diagnosis in the SLC6A8 database (www.LOVD.nl/SLC6A8) and were not identified in 276 control chromosomes (Betsalel et al. 2011). All mutations, except the c.541T>C; p.(Cys181Arg) missense mutation, are truncating mutations and thus should be considered pathogenic mutations. The molecular data of patients 2 and 5 were previously reported (Bizzi et al. 2002; Betsalel et al. 2011). The c.263-1G>C results in erroneous splicing and also in a truncated protein p.Gly88Leu108del (Betsalel et al. 2011). However, also the missense mutation is also considered a pathogenic mutation since it results in the replacement of a conserved amino acid (Table 1). In all patients an uptake assay in fibroblasts showed no significant uptake of Cr uptake compared to normal controls (data not shown). A random pattern of X chromosome inactivation was found in the two female patients' leucocytes. A skeletal muscle biopsy was performed in patient 1, in order to rule out another cause of myopathy. Muscle histology was normal.

Methods

GAA and creatine were determined in plasma and urine by tandem mass spectroscopy as previously described. (Mercimek-Mahmutoglu et al. 2009)

DNA was isolated from white blood cells or cultured fibroblasts, collected from patients and their parents after informed consent. The coding region and the adjacent splice sites of *SLC6A8* human gene were analyzed by direct sequence analysis and creatine uptake studies were performed in patients 1, 4, 5 and 6 fibroblasts as previously reported (Salomons et al. 2001).

Molecular studies were performed in the two female patients in leucocytes to investigate the pattern of X chromosome inactivation using the method described earlier (van de Kamp et al. 2011).

All patients have been treated for 42 months with oral Cr (400 mg/Kg/day) alone for 6 months, then by Cr combined to its natural precursors L-Arg (200 mg/Kg/day) and L-Gly (200 mg/Kg/day) for 12 months and finally L-Arg (200 mg/Kg/day) and L-Gly (200 mg/Kg/day) without Cr for 24 months.

Patients' outcome was evaluated by clinical and neuropsychological assessment using where possible age-adapted cognitive tests. Brain MRI with MRS (TE 144) was the main endpoint of the study. It was performed for all patients, before and every 6 months after the onset of the therapeutic trials during the first 24 months then yearly. Safety was assessed by monitoring kidney and tubular function (plasma urea and creatinine, microalbuminuria, proteinuria, hematuria) were monitored prior and during treatment for creatine toxicity.

Informed consent was obtained from each patient prior to entering the study. The study has been approved by our institutional ethics committee.

Results

No side effect was reported other than a rapid weight gain within the first months of treatment of 1 to 5 Kg in most patients related with an increase in patients' muscular mass. Body mass index remained within the normal range in all patients. Kidney and tubular function markers remained unchanged.

The cognitive and behavioural outcome of the patients is described in Table 1. None of the patients showed improvement of their cognitive status. In P1 a formal cognitive evaluation was impossible to perform before or after treatment. In P2 and P3 behaviour disturbance deteriorated and did not allow controlling their cognitive levels. Only P5 showed some improvement as she experienced fewer hallucination episodes as reported by her parents and by her psychiatric institution's professionals. Epileptic seizures persisted in patient 4 but did not relapse in patients 1 and 3.

By contrast, muscular symptoms were dramatically improved in patients 1 and 3 who increased their muscular mass, improved their balance and walking and developed almost normal gross motor skills during the treatment period (Table 1).

No improvement of brain creatine in MRS has been observed in any patient including the two female patients (Fig. 2).

Urinary Cr increased in all patients indicating good compliance to treatment; GAA levels remained normal. CK were normal for all patients and renal function biochemical markers remained unchanged.

Discussion

We report on six patients, four males and two females, presenting with a severe form of CTP deficiency. Clinical presentations include mild to severe mental retardation (6/6), associated with psychiatric symptoms (5/6: autistic behaviour, chronic hallucinatory psychosis), seizures (2/6) and muscular symptoms (2/4 males). Because CTP deficiency is the most frequent cause of Cr defects, our observations reinforce the conviction of including creatine metabolic defects to the screening of patients, males but also females, with non-specific mental retardation, associated to autistic spectrum disorders or other psychiatric symptoms, seizures or muscular hypotonia. A severe CTP phenotype is possible in females (van de Kamp et al. 2011), probably depending of the mosaic expression of the mutant and wild-type alleles. However, skewed X inactivation was not found in our two female patients in peripheral blood. Normal urinary creatine results in one of these two patients shows that the diagnosis of CTP deficiency in female

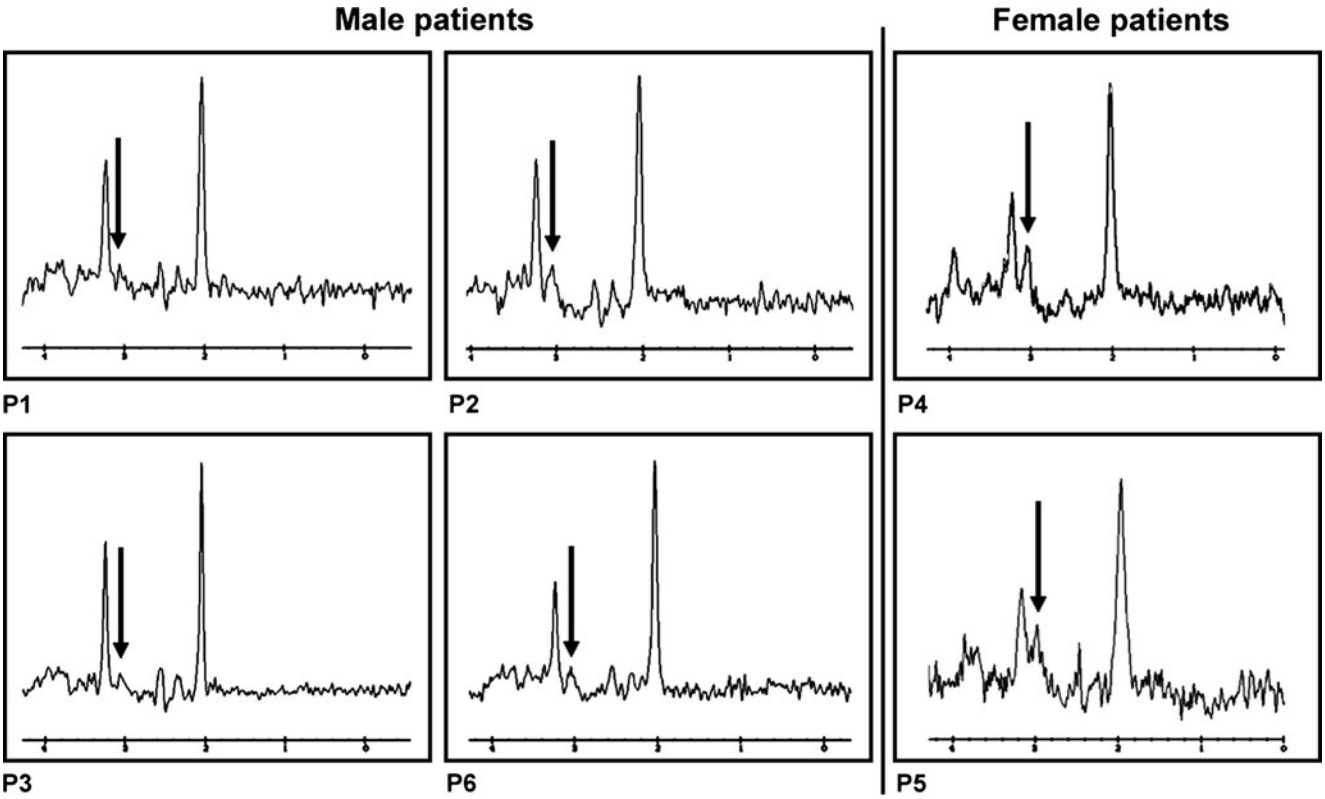

Fig. 2 Brain magnetic resonance spectroscopy scans (MRS) from all patients (P1-P6) at the end of the study. The black arrows depict the creatine peak position. No significant changes in MRS were observed

patients could be missed by urinary screening. MRS is thus considered as a better diagnostic tool for patients with CTP defect, displaying a very low or absent Cr peak in the brain parenchyma of males, and a decreased Cr peak in females. However it has been reported in a few female symptomatic carriers brain Cr levels within the normal range (van de Kamp et al. 2011). Therefore sequence analysis is the best diagnostic tool for symptomatic females when Cr deficiency is suspected.

In five new patients we identified three novel mutations not reported so far, including a mutation identified twice in two male patients.

The discovery of Cr defects has revealed the importance of blood Cr supply for normal brain and muscle function, even though AGAT and GAMT are widely expressed in the central nervous system (Braissant et al. 2007) where Cr synthesis can occur. Cr may play an important role in the promotion of neuronal differentiation during development of neuronal cells (Andres et al. 2005a, b), and it may act as cotransmitter modulating postsynaptic GABA receptors (Almeida et al. 2006).

We firstly tried to administrate high doses of Cr to our patients, particularly in the two female patients hypothesizing that they may benefit from a partial uptake given the simultaneous expression of the mutant and wild-type alleles (which was shown in peripheral tissues for P5). Unfortunately this therapeutic trial had little or no effect on neurological and psychiatric features and failed to increase the Cr peak at MRS, while it dramatically improved muscular symptoms in two male patients, and increased muscular mass in all patients.

Due to the lack of response to Cr, other therapeutic strategies have been proposed, such as L-Arg alone or combined to L-Gly (Leuzzi et al. 2008). The latter are the precursors of Cr synthesis and are transported independently from SLC6A8 into the CNS. The hypothesis here is that L-Arg and L-Gly can be used by intra-cerebral AGAT and GAMT (Braissant et al. 2007) for Cr synthesis. Because L-Gly acts as a substrate stimulating the synthesis of Cr at the level of astroglial cells (Dringen et al. 1998), their supplementation appeared an attractive therapeutic option aimed at stimulating the endogenous synthesis of Cr. However we did not observe any effect of this treatment on the neurological and psychiatric features of our patients, similarly to what was previously reported in another series of four patients (Fons et al. 2008) and found no change on creatine brain levels in brain-MRS after treatment with L-Arg and L-Gly. We recognize that in some patients repeating cognitive assessments was difficult due to their behavioral disturbances and the latter could limit the interpretation of the results on the outcome in these patients. However the unchanged brain MRS that was the main endpoint of the study is in line with these results.

Interestingly, two recent observations report improvement of intellectual capacities, behaviour and epilepsy, as well as brain Cr levels in single case patients after supplementation with L-Arg alone (Chilosi et al. 2008) or in combination with Cr and L-Gly in a female patient (Mercimek-Mahmutoglu et al. 2010). In the first paper the authors concluded that it was difficult to separate the effects due to the patient's natural history from those due to L-Arg supplementation and in both papers the MRI changes were very mild and remain far from normal. Also epilepsy deteriorated in P4 during the treatment period whereas it improved dramatically in the patient reported by Mercimek-Mahmutoglu et al. (Mercimek-Mahmutoglu et al. 2010) treated with a similar protocol using L-Arg, L-Gly and Cr. The molecular findings cannot explain the different therapeutic responses in these patients as at least one "responsive" patient had a nonsense mutation. Hypotheses to explain the absence of response in our and in other patients could be that AGAT expression in brain is too low to significantly increase the amount of cerebral Cr even after L-Arg (and L-Gly) supplementation, or that the Cr transporter is also needed for the transport of Cr in neurons. Indeed, recent data suggest that AGAT and GAMT are rarely co-expressed in one cell and the Cr transporter may be needed for GAA uptake from AGAT- to GAMT-expressing cells (Braissant et al. 2010). Thus even if GAA is formed, it may not be available for further Cr metabolism since GAA may not enter the cells that express GAMT. The dose of 200 mg/kg/d for L-Arg was defined based on our current practice in patients with urea cycle defects where arginine synthesis is impaired, even though higher doses where used in other studies but for shorter periods of time {300 mg/kg/d for 12 months, (Chilosi et al. 2008), 400 mg/kg/d (Fons et al. 2008) for 24 months, 450 mg/kg/d (Mercimek-Mahmutoglu et al. 2010) for 12 months}. We considered L-Arg doses over 400 mg/kg/d to be very high with a potential risk of toxicity if used for prolonged periods of time as high arginine may increase nitric oxide synthesis (Palmer et al. 1988).

Moreover all patients had their plasma levels of arginine and glycine monitored regularly that remained within the normal range when measured before the following dose but increased up to two times above the upper limit for age 2 h after each dose. In three additional patients with CTP, we used L-Arg combined to L-Gly up to 400 mg/kg/day for 6 months with no clinical response and no change on brain MRS (data not shown). Finally the absence of response in our female patients and despite a random X skewing seems surprising but in line with the severe phenotype of these patients.

In conclusion, CTP defect is a cause of severe mental retardation in males and females, and may present with a myopathic phenotype in males. In our patients Cr supplementation alone or with its precursors L-Gly and L-Arg

showed benefit only in the muscular symptoms of the disease and no improvement in the cognitive and psychiatric manifestations of male and female CTP deficient patients. Novel therapeutic strategies are required that may include Cr analogs or alternative pathways of transport independent from SLC6A8.

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