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Key Words:	Creatine Deficiency Syndrome, GAMT gene, SLC6A8 gene, Metabolic stress, Reactive oxygen species, Mutation Analysis

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DEFINING THE PATHOGENICITY OF CREATINE DEFICIENCY SYNDROME

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ABSTRACT

This work examined nine patients with creatine deficiency syndrome (CDS), six with a creatine transport (CRTR) defect and three with a GAMT defect. Eleven nucleotide variations were detected: six in *SLC6A8* and five in *GAMT*. These changes were analysed at the mRNA level and specific alleles (most of which bore premature stop codons) were selected as nulls since they provoked nonsense-mediated decay activation. The impact of these CDS mutations on metabolic stress (ROS production, p38MAPK activation, aberrant proliferation and apoptosis) was analysed in patient fibroblast cultures. Oxidative stress contributed towards the severe form of CDS, with increases seen in the intracellular ROS content and the percentage of apoptotic cells. An altered cell cycle was also seen in a number of CRTR and GAMT fibroblast cell lines (mostly those carrying null alleles). p38MAPK activation only correlated with oxidative stress in the CRTR cells. Based on intracellular creatine levels, the contribution of energy depletion towards metabolic stress was demonstrable only in selected CRTR cells. Together, these findings suggest that the apoptotic response to genotoxic damage in the present CDS cells may have been triggered by different cell signalling pathways. They also suggest that reducing oxidative stress could be helpful in treating CDS.

Keywords: Creatine Deficiency Syndrome, CDS, GAMT, SLC6A8, Metabolic stress, Reactive oxygen species, Mutation Analysis

INTRODUCTION

Cell energy demand and supply are balanced and tightly regulated to ensure economic and efficient energy use. Cells with sudden high energy demands, such as neurons, can increase their rate of ATP hydrolysis by several orders of magnitude within seconds of the need arising, yet intracellular ATP levels remain constant. Via reversible phosphorylation by creatine kinase (CK), creatine (Cr) forms part of a high-energy ADP-to-ATP phosphate buffering system (Wyss and Kaddurah-Daouk, 2000), allowing the shuttling of high-energy phosphates from the mitochondria to the cytoplasmic sites where they are required. Cr is also a potential neuromodulator or co-transmitter in the central nervous system (CNS), regulating the activity of post-synaptic receptors such as GABA_A (Almeida, et al., 2006b). In mammals, Cr is normally transported through the blood and taken up by the Cr transporter, a 12-transmembrane-spanning protein (known as CT1 or CRTR) that belongs to a superfamily of Na⁺/Cl⁻-dependent neurotransmitter transporters (SLC6) (Snow and Murphy, 2001). This transporter is encoded by the *SLC6A8* gene located at Xq28. Owing to the spontaneous conversion of Cr to creatinine (Crn), which is excreted in the urine, the Cr pool must be maintained via daily nutritional intake and/or *de novo* synthesis. Endogenous synthesis occurs in the cytosol compartment and involves two enzymes, L-arginine:glycine amidinotransferase (AGAT, EC 2.1.4.1), which uses arginine and glycine as substrates and yields the intermediate guanidinoacetate (GAA), and S-adenosyl-L-methionine:N-guanidinoacetate methyltransferase (GAMT, EC 2.1.1.2), which converts GAA to Cr in the presence of S-adenosyl-L-methionine.

Proton magnetic resonance spectroscopy (¹H-MRS) has revealed the brains of some patients with mental retardation to have little or no Cr. This discovery led to the identification of three

congenital disorders of Cr metabolism, collectively known as creatine deficiency syndrome (CDS). Two of these disorders involve problems of Cr biosynthesis caused by deficiencies of the enzymes AGAT (MIM# 602360) (Bianchi, et al., 2000) and GAMT (MIM# 601240) (Stockler, et al., 1994), while the third is an X-linked inherited deficiency of the creatine transporter (CRTR deficiency; MIM# 300036) (Salomons, et al., 2001). The measurement of GAA and the Cr/Crn ratio in body fluids can be used to identify CDS patients; patients with GAMT deficiency show high GAA concentrations compared to controls, while patients with AGAT deficiency have low levels. Those with a CRTR disorder show normal GAA concentrations but a significantly increased urinary Cr/Crn ratio.

To date, more than 100 patients with a CDS have been reported, mainly with a CRTR or GAMT deficiency (Almeida, et al., 2006a; Dhar, et al., 2009). Mutation analysis of *SLC6A8* and *GAMT* has recorded the existence at least 50 different nucleotide changes, mostly point mutations or small deletions (HGVS database) (<https://portal.biobase-international.com/hgmd/pro/start.php>). However, as in other diseases, it has been difficult to establish a clear correlation between the mutation type and the severity of the clinical phenotype.

Patients with CDS suffer motor dysfunctions, developmental delay, mental retardation, epileptic crises, autistic behaviour and speech problems, highlighting the importance of Cr metabolism and transport in the CNS (Beard and Braissant, 2010). Owing to the neurotoxic effects of GAA accumulation in the grey matter (Neu, et al., 2002), patients with GAMT deficiency also suffer dystonic hyperkinetic movement disorder.

Since CDS can result in a reduction in total cellular ATP, it might also affect intracellular ATP pools via CK-mediated ATP/ADP shuttling. Cr depletion in the brain has been associated with the disruption of neuronal function and changes in mitochondrial structure (Andres, et al., 2008).

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Recent studies have proposed crosstalk to occur between metabolic stress and the Cr pathway. Neurodegenerative diseases, such as Alzheimer disease, Parkinson’s disease and amyotrophic lateral sclerosis all have the hallmark of mitochondrial dysfunction resulting in ATP depletion and overproduction of mitochondrial reactive oxygen species (ROS). Cr supplementation, however, has a protective antioxidant effect in these conditions, at least in animal models. Corroborating this possible crosstalk between metabolic stress and the Cr pathway, a new role has been reported for GAMT activity as a p53-inducible modulator of apoptosis, critical for the adaptive response to nutrient stress (Ide, et al., 2009).

The present study reports the characterisation of nine patients with CDS showing Cr metabolism or transport defects at the biochemical and genetic level, and explores the impact of their specific genotypes on the variables of cellular metabolic stress, including ROS production and apoptosis.

MATERIALS AND METHODS

Patients

The study subjects were nine unrelated Spanish patients with CDS, seven males and two females, ranging from three to 47 years of age. Patients were identified either by their clinical symptoms and abnormal ¹H-MRS scans, or during the investigation of a possible inborn error of metabolism. Following an established flowchart for the differential diagnosis of CDS (Almeida, et al., 2004), urine and plasma Cr and GAA levels were analysed by HPLC/MS/MS as a first step. An increased urine GAA concentration (>150 mmol/mol Crn) and the Cr/Crn ratio (>1.5)

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3 were used as biochemical markers of GAMT and CRTR defects respectively. Six patients were
4 identified as having a possible defect in CRTR activity, and three with a possible defect in
5 GAMT activity. Primary fibroblast cell cultures were used to confirm CRTR and GAMT
6 defects. Creatine transport was measured using $[4\text{-}^{14}\text{C}]\text{-Cr}$ ($0.2\text{ }\mu\text{Ci/mL}$) at physiological
7 concentrations ($3\text{-}125\text{ }\mu\text{M}$) (Alcaide, et al., 2010). After incubation of the cells with $[^{13}\text{C}_2]\text{-}$
8 guanidinoacetate and $[^2\text{H}_3]\text{-S-adenosylmethionine}$, GAMT activity was determined by analysing
9 the final product of the reaction $[^{13}\text{C}_2\text{-}^2\text{H}_3]\text{-Cr}$ by HPLC-MS/MS (Verhoeven, et al., 2004).
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20 Clinical assessments were made for all patients by at least one of the authors. Written informed
21 consent to be included in the study was obtained from all subjects (or their legal guardians), or
22 in the case of minors from their parents/legal guardians. Ethical approval for the use of human
23 fibroblasts in the present study was obtained from the Ethics Committee of the *Universidad*
24 *Autónoma de Madrid*.
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36 Culture conditions

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38 Skin fibroblasts from patients and controls were cultured according to standard procedures in
39 minimal essential medium (MEM) supplemented with 1% glutamine, 10% foetal calf serum
40 (FCS) and antibiotics in a humidified atmosphere containing 5% CO_2 . Control cell lines
41 (GM03348, GM08333, GM08429, GM08680, GM09503) aged 2-10 years were obtained from
42 the Coriell Institute for Medical Research, NIGMS Human Genetic Cell Repository, USA. Skin
43 fibroblasts from three healthy individuals aged 20-41 years were also included as controls. Most
44 of the cells used in this study were in their 7th-11th passage. Unless otherwise stated, assays were
45 performed when the cultures reached at least 80% confluence.
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Mutation analysis

Fibroblast cell lines, dried blood spots and/or whole blood were used as sources of DNA and/or RNA. Mutation analysis was performed by direct sequencing of overlapping PCR fragments obtained after amplification of the entire coding sequence of *GAMT* or *SLC6A8*. The mutations were further confirmed by sequencing both strands of corresponding exons and their flanking intronic sequences.

Total mRNA was isolated using the Tripure Isolation Reagent (Roche Applied Science, Indianapolis, IN, USA) adhering to the manufacturer’s protocol, followed by two-step RT-PCR using oligodT as a primer and employing the SuperScript III First-Strand enzyme (Invitrogen, Carlsbad, CA, USA). Genomic DNA was isolated as previously described (John, et al., 1991). Amplifications of genomic exons were performed using the FastStart kit (Roche Applied Science). Primers to amplify cDNA or genomic sequences were designed according to the cDNA GenBank sequences NM_000156.4 for *GAMT* and NM_005629.3 for *SLC6A8*, or gene GenBank sequences NT_011255 for *GAMT* and NT_025965 for *SLC6A8*. All primer sequences are available upon request. All PCR products were sequenced employing BigDye Terminator v.3.1 mix (Applied Biosystems Foster City, CA, USA) following the manufacturer’s protocol, purified in Performa V3 96-well short plates (EdgeBio, Gaitherburg, MD, USA), and analysed by capillary electrophoresis using an ABI Prism 3700 Genetic Analyzer (Applied Biosystems). DNA mutation numbering was based on the cDNA reference sequences taking nucleotide +1 as the A of the ATG translation initiation codon. The nomenclature used follows the recommendation of the Human Genome Variation Society (<http://www.hgvs.org/mutnomen/>) (Dunnen and Antonarakis, 2000).

***In silico* analysis**

Conservation of the mutated residues was assessed by alignment of orthologous and human protein sequences using ClustalW software (<http://www.ebi.ac.uk/clustalw/>) (Larkin, et al., 2007). The putatively damaging effects of the predicted amino acid substitutions were assessed using the PolyPhen programme (<http://genetics.bwh.harvard.edu/pph/>) (Ramensky, et al., 2002); the results are given as “benign”, “possibly damaging”, “probably damaging” or “unknown”. The bioinformatic analysis of potential splicing aberrations was performed using Human Splicing Finder (HSF) v.2.4 software: <http://www.umd.be/HSF/> (web-based) (Desmet, et al., 2009).

Real-time PCR quantification

SLC6A8 and *GAMT* mRNA expression levels were assessed in patient and control cell lines by performing qRT-PCR, using ProbeFinder software (Roche Applied Science) for the optimal design of the real-time PCR primers, and the Universal Probe Library (Roche Applied Sciences) for probe selection. Total RNA obtained as described above was retro-transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems) and PCR-amplified using the Fast-Start Taqman Probe Master kit (Roche Applied Science). The *GAPDH* mRNA expression level was used as an endogenous control. Real-time PCR and analysis were performed in an ABIPRISM 7900HT Genetic Analyzer (Applied Biosystems) using the SDS2.1 programme. RNA integrity was analysed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Relative quantification of gene expression was analysed by the comparative threshold cycle (Ct) method

(Livak and Schmittgen, 2001; Schmittgen and Livak, 2008); ΔC_T was calculated as the difference in C_T between the target gene and the housekeeping gene for each sample. The following formula was used to calculate the relative amount of transcripts in patients with respect to controls: $\Delta\Delta C_T = \Delta C_T$ (patient) - ΔC_T (control) for each gene. The fold-change for each gene in each patient relative to the control was calculated as $2^{-\Delta\Delta C_T}$.

ROS and apoptosis assays

For ROS and apoptosis assays, skin fibroblasts from three controls and the nine CDS patients were cultured as previously described (Richard, et al., 2007). The cells were detached by trypsinisation for 1 min, which was immediately stopped by adding complete medium. ROS levels (H_2O_2) were monitored using the H_2DCFDA fluorescence probe (2,7-dichlorodihydrofluorescein diacetate; Molecular Probes, Inc., Eugene, OR, USA) (5 μM) by flow cytometry after 30 min of incubation at 37°C in the dark.

Apoptotic cells were identified and quantified by flow cytometry using the Annexin V-DY634 apoptosis detection kit (Immunostep Research, Salamanca, Spain). Fibroblasts harvested by trypsinisation as mentioned above were incubated with 2.5 μL of Annexin V-DY634 for 15 min at room temperature in the dark.

Propidium iodide (0.001%; SIGMA, St Louis, MO, USA) was used to detect dead cells. Samples were analysed using a FACSCalibour apparatus with two solid-state lasers (488 and 633 nm) (Becton Dickinson, Franklin Lakes, NJ). A minimum of 10,000 individual events were recorded. Data were acquired and analysed using Cell Quest-Pro (Becton Dickinson) and FlowJo software (FlowJo, Ashland, OR, USA) respectively.

Flow cytometric analysis of the cell cycle and nuclear DNA content

Cells were grown to a density of 80% and the culture medium replaced by MEM with 0.5% FCS for synchronisation over 48 h. The cells were then transferred back to MEM +10% FCS for 24 h followed by harvesting and counting. 1.5×10^6 cells were fixed in 70% ethanol for at least 18 h at -20°C , and treated with PI/RNase Staining Buffer (BD Pharmingen, San Diego, CA, USA) for 30 min at 37°C in the dark (Kues, et al., 2000; Nishikiori, et al., 2008). The DNA content was analysed by flow cytometry using a BD FACSCanto II flow cytometer (BSBioscience, San Jose, CA). At least 10,000 events were acquired per sample. Data were acquired using FACSDiva software and the relative percentages of cells in the sub-G0/G1 (apoptotic cells), G0/G1, S and G2/M stages determined using ModFit LT v.2.0 TM software (Verity Software House Inc., Topsham, ME, USA).

Western blot analysis

A total of 4×10^5 fibroblast cells of each line were placed in 6-well plates 24 h prior to protein analysis. Cells were lysed with 50 μl /well of ice-cold lysis buffer (1% Triton X-100, 10% glycerol, 150 mM NaCl, 10 mM Tris-HCl pH 7.5, protease and phosphatase inhibitor cocktail tablets [Roche Applied Science]). After centrifugation, the protein content of the supernatants was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA) using BSA as a standard. Samples were loaded onto 4-12% NuPAGE Novex Bis-Tris minigels (Invitrogen, Carlsbad, CA, USA) and blotted onto nitrocellulose membranes using the iBlot Dry Blotting system (Invitrogen). Ponceau staining was used to verify equal protein loads. Western blotting

was performed using the phospho-p38 MAP kinase (Thr180/Tyr182) polyclonal antibody (Cell Signaling Technology, Inc, Danvers, MA, USA) and p38 polyclonal antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA). The secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (Santa Cruz Biotechnology); these were detected using the Enhanced Chemiluminescence System (GE Healthcare, Berkshire, UK). Protein quantification was performed using a calibrated densitometer GS-800 (Bio-Rad Laboratories).

Fibroblast creatine content

Confluent cells from CDS patients and controls were harvested by trypsinisation, pelleted by centrifugation and stored at -80°C. Cell pellets were resuspended in 0.2 mL of PBS, frozen and thawed three times in liquid nitrogen, and centrifuged (14,500xg for 5 min). The supernatant was used for Cr quantification by HPLC/MS/MS, employing stable isotope-labelled Cr as an internal standard, and for protein determination by the Lowry method. The Cr content was expressed in pmol/μg protein.

Statistical analysis

ROS, apoptosis, cell cycle stage and nuclear DNA contents were determined in at least three independent experiments, each performed in triplicate. Values were expressed as means ± SD unless otherwise stated. All *p* values were two-tailed. Significance was set at *p*<0.05.

RESULTS

Biochemical data and mutation analysis of CDS patients

The relevant biochemical and clinical information of the patients with a defect in either *SLC6A8* (CRTR patients) or *GAMT* (GAMT patients) is included as Supporting Information (Supp. Table S1 and Supp. Table S2).

Eleven nucleotide sequence variations were identified that presumably lead to loss of function: six in *SLC6A8* and five in *GAMT*. Of these, seven have been previously (and specifically) reported in Spanish patients (Alcaide, et al., 2010; Fons, et al., 2009; Item, et al., 2004; Sempere, et al., 2009a; Sempere, et al., 2009b). Table 1 shows their distributions in the two genes. The rest correspond to allelic variants previously described in other populations (Bizzi, et al., 2002; Clark, et al., 2006; Item, et al., 2004). Most of these changes affect residues highly conserved between the corresponding human proteins and their homologues, as determined by BLAST analysis and comparisons made against sequences available in the NCBI nucleotide database (www.ncbi.nlm.nih.gov) using ClustalW software. Table 1 shows that all missense mutations were predicted to be “damaging” in PolyPhen analysis.

In *SLC6A8*, the distribution of nucleotide changes included three nucleotide substitutions (a nonsense change and two missense mutations) and three in-frame small deletions. Most of the changes identified affected residues in the transmembrane domains of the protein.

Given the hemizygous condition of the *SLC6A8* mutants, Cr transport was directly measured at physiological substrate concentrations in patient fibroblasts to characterise the effect of the mutations. All patient fibroblasts showed a significant reduction in Cr uptake compared to control cells (<20%) (data not shown).

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In *GAMT*, the mutant alleles involved two missense and two nonsense changes (one of each in heterozygous fashion in patients P7 and P9), and one small deletion in a homozygous fashion in patient P8. Three of these changes have previously been identified in the Spanish population. The remaining mutations corresponded to the p.W20S mutation, which appears to be very common in Portugal (Almeida, et al., 2007), and to p.W174X, which has been described in other populations (Item, et al., 2004).

Genetic analysis of the available family members (Supp. Table S1 and Supp. Table S2) showed that *GAMT* changes co-segregated in the respective families. However, controversial data were obtained for the *SLC6A8* gene; co-segregation could only be demonstrated for the change c.1079_1081delTCT. Similar results have been previously reported for other *SLC6A8* mutants. Although the most plausible explanation would be that these mutations arose *de novo* as described in (Battini, et al., 2007), somatic mosaicism cannot be excluded (Betsalel, et al., 2008).

As the mutational spectrum identified included several changes leading to premature termination codons (PTCs) capable of inducing nonsense-mediated mRNA decay, the expression profiles of *SLC6A8* and *GAMT* were examined (Fig. 1). The mRNA levels (quantified by qRT-PCR) of the *SLC6A8* alleles and c.145delT *GAMT* allele appearing in homozygous fashion in patient P8 were compared to those of the control fibroblast cell lines (10-40 years old). Cell lines from *GAMT* patients bearing the nonsense changes in heterozygous fashion were also included. The results showed a clear decay of the PTC-bearing alleles analysed; this decay, however, was rescued when cells were treated with the translation inhibitor emetine (data not shown). Interestingly, the relative abundance of the *SLC6A8* allele carrying the nucleotide variation c.1079_1081delTCT (P3) was also significantly reduced compared to controls. Some exonic mutations may alter normal mRNA processing and lead to the synthesis of aberrant mRNA, which may or may not be

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3 degraded. Bioinformatic modelling can help to assess the potential effect of such variants on
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5 splicing. In this work, HSF software was used to predict the effects of mutations on splicing
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7 signals or to identify splicing motifs. According to the HSF prediction, the change
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9 c.1079_1081delTCT disrupts a low score potential acceptor splice site (score: 66.33→20.51) and
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11 alters enhancer motifs recognised by SC35, SRp40 and SRp55 proteins, either breaking the site
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13 or creating a new mutant motif. However, no aberrant transcript could be identified after
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15 sequencing.
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20 21 22 23 **Metabolic stress variables**

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25 ROS are products of normal metabolism, and depending on their concentration can be beneficial
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27 or harmful to cells (Circu and Aw, 2010). A number of pathologies can result from oxidative
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29 stress-induced apoptosis following increases in ROS (Ma, 2010). To explore the impact of the
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31 CDS mutant alleles on metabolic stress variables, intracellular ROS contents, apoptosis levels
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33 and the progression of the cell cycle were examined in all nine CDS cell lines.
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38 39 **Reactive oxygen species**

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41 The comparison of patient and control fibroblasts showed those of patients P1 and P3 (CRTL
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43 patients), and P7 and P9 (GAMT patients), to have significantly increased ROS levels (1.5-2.4-
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45 fold, with those of P3 the highest) (Fig. 2).
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49 The activation of a protein belonging to the mitogen-activated protein kinase (MAPK)
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51 superfamily, p38 kinase, was also analysed by immunoblotting to compare its activation with the
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53 ROS levels detected in CDS patients. The expression pattern of the phosphorylated p38 protein
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55 was individually normalised by the protein expression of total p38 protein, which includes both
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the phosphorylated and non-phosphorylated forms. The expression of phospho-p38 was most strongly detected in the cell lines of the CRTR patients (P1 and P3), i.e., those with the highest ROS levels (Fig. 3). Quantitative densitometry showed a >10-fold higher expression for phospho-p38 in the P1 and P3 cell lines compared to controls ($p<0.05$). The CRTR cell lines with the lowest ROS contents (i.e., cells from P2, P4 and P6) showed the weakest activation of this kinase (below or equal to that seen in the controls). The results obtained for p38 protein activation correlated with the ROS levels in the CRTR patients' cell lines; however there was no clear correlation between phospho-p38 expression and ROS levels in the GAMT cell lines.

Apoptosis in CDS cell lines

To address the possibility that elevated ROS levels might induce apoptosis in the patient cell lines, the percentage of annexin V-positive cells was investigated by flow cytometry using dual staining with annexin V and PI. This method identifies the apoptotic mode of cell death: annexin V-positive/PI-negative cells were considered early apoptotic cells and annexin V-positive/PI-positive as late apoptotic or necrotic cells. An increase in annexin V-positive/PI-negative (1.3-2-fold) and annexin V-positive/PI-positive (1.25-2.5-fold) cells was seen for patients P1, P3, P7 and P9 (Fig. 4). The greatest binding of annexin V was seen in cells from patients P3 and P9. These results indicate that fibroblasts from CDS patients with increased ROS levels also show an increase in both early and late apoptosis.

Progression of the cell cycle

Apoptotic cells have reduced DNA contents and can therefore be recognized as cells with low DNA stainability (sub-G0/G1 peak) (Nicoletti, et al., 1991; Umansky, et al., 1981). Figure 5 shows that most of the CDS cell lines had an altered cell cycle distribution pattern compared to the control lines (including fibroblasts from adults). Cell lines from patients P1, P3, P7 and P9 showed a very significant percentage of cells at the sub-G0/G1 stage, as seen from their reduced DNA content (less fluorescence and diminished forward light scatter relative to the peak for cells at G1 [14 ± 2 for P1, 18 ± 11 for P3, 15 ± 0.9 for P7 and 18 ± 2.7 for P9], indicating apoptosis. A significant accumulation of cells in the G2/M phase was also detected, delaying the transition to G1 in most of the patient cell lines.

Intracellular creatine content

To correlate the apparent different behaviours of the patient cell lines in the context of metabolic stress with their capacities to maintain Cr homeostasis, an MS-MS method was used to measure the intracellular Cr levels in fibroblasts grown under standard conditions. Table 2 shows that all CRTR cell lines, irrespective of their genotype, suffered a severe reduction in their intracellular Cr content. However, in all the GAMT cell strains studied, Cr levels were similar to control values, indicating that Cr was efficiently taken up from the culture medium.

DISCUSSION

Genetic variability

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A total of 11 variants (four missense mutations, three nonsense mutations, three in-frame deletions and 1 frameshift change) were identified in the present cohort of six CRTR and three GAMT patients. The supposed pathogenicity of the missense changes and the small in-frame deletions was based on the conservation of the mutated residue across orthologous proteins, and on *in silico* predictions. Most of mutations found affected conserved residues, and all belong to stretches of conserved amino acid. The PolyPhen bioinformatic prediction suggested all the missense changes analysed to be potentially harmful to the function of the corresponding proteins. Special importance was associated with the *GAMT* p.T136M change, which was predicted to lead to a change in the hydrophobicity of a residue at the core of the protein. However, its pathogenicity needs to be tested in overexpression studies (Almeida, et al., 2007; Rosenberg, et al., 2007). The frameshift and nonsense variants were highly likely to be pathogenic since they result in dysfunctional and perhaps harmful proteins. Moreover, the drastic reduction of *SLC6A8* or *GAMT* transcripts in the CRTR and GAMT cell lines carrying nonsense or frameshift changes was consistent with the eliciting of nonsense-mediated mRNA decay. An exception to the reading frame rule was obtained for the small in-frame deletion c.1079_1081delTCT which provoked a significant reduction in *SLC6A8* mRNA in the P3 cell line. Although the bioinformatic prediction suggested this could be explained by a change in the splicing control sequences, the experimental data reflected mRNA instability unassociated with the generation of aberrant transcripts containing PTCs.

Correlating genotypes with phenotypes is not straightforward in CDS. Patients with genetic CDS usually show important neurological symptoms, but the lack of standardised criteria for classifying the severity of disease limits any associations that might be made. In the present

cohort, the mildest disease presentation was seen in P4 patient, who carried p.A404V, the only change affecting a non-conserved residue of the CRTR protein (Alcaide, et al., 2010).

Creatine deficiency and metabolic stress

There is increasing evidence that oxidative stress and apoptosis are closely associated phenomena that might explain the neuropathological aspects of several metabolic and neurodegenerative diseases. At normal concentrations, ROS play a role in signal transduction, including that involved in proliferation. However, an imbalance between ROS generation and the capacity of antioxidants to neutralise them can result in the disruption of the cellular redox status, leading to oxidative stress (Circu and Aw, 2010).

The present results show, for the first time, an overproduction of ROS in two CRTR (P1 and P3) and two GAMT (P7 and P9) patient fibroblast cell lines. These cell lines also showed increased apoptotic rates, measured as the percentage of annexin V-positive cells. This suggests that elevated ROS levels might induce apoptosis in CDS cell lines, as previously suggested for other conditions (Benhar, et al., 2002; Kim and Wong, 2009; Ma, 2010).

A loss of the redox control of the cell cycle could lead to aberrant proliferation (Sarsour, et al., 2009). Cell cycle progression is regulated by checkpoint controls that protect the integrity of the genome (Hartwell and Weinert, 1989). If cycling cells are damaged they usually have the capacity to pause temporarily at G1, S, or G2, repair the damage (if possible), and then re-enter the cell cycle. When damage is severe, however, cells may undergo programmed apoptosis (Shackelford, et al., 2000). In the present work, a significant percentage of cells were found to be at the sub-G0/G1 stage in the cells of patients P1 and P3 (CRTR), and in those of P7 and P9

(GAMT). The significant inhibition of G2M/G1 transition, identified in most of the CDS cell lines, indicates the position of a possible checkpoint.

Replicative cell division is an energetically demanding process. To successfully undergo it, cells must have an adequate supply of essential nutrients to support both ATP production and macromolecule synthesis (Jones, et al., 2005). In the present work it was first hypothesized that the altered cell cycling pattern observed in the CDS strains might reflect energy depletion as a result of the reduced Cr content. However, when intracellular Cr levels were measured, only cells from CRTR patients showed a drastic reduction in Cr (Cr concentrations were normal in the GAMT cells). In addition, intracellular Cr levels did not always correlate well with the cellular stress phenotype in the CRTR patients - P2, P5 and P6 manifested a mild (or no) cellular stress phenotype. These data strongly support that Cr uptake is the main pathway involved in maintaining Cr homeostasis in fibroblasts, and underscore the role of Cr metabolism in the response to cellular stress. Indeed, ROS damage observed in the P7 and P9 GAMT cells cannot be explained by reduced high energy phosphate reserves since the intracellular Cr levels were in the same range as the controls. Previous research in animals showed that GAA inhibits $\text{Na}^+\text{-K}^+$ -ATPase activity, which might be mediated by nitric oxide formation (Zugno, et al., 2004). Reduced brain antioxidant capacity related to GAA accumulation is also reported in the rat striatum, indicating the possible involvement of oxidative injury, offering an explanation for the pathogenic mechanism of brain damage in patients with GAMT deficiency (Zugno, et al., 2008).

p38 MAPK was found to have a different activation pattern in the CRTR and GAMT cell lines, suggesting that the apoptotic response to genotoxic damage in these cells might be triggered by different signalling pathways. In fact, the activation of p38 MAPK, commonly related to stress response signals such as oxidative damage, was particularly noticeable in the CRTR patient cell

lines that showed the highest ROS and apoptosis levels. However, the activation of this protein was not detected in the GAMT patient fibroblasts that showed an increase in ROS and apoptosis. These promising results in GAMT cell lines will be followed up with further studies focusing on the components of the signalling pathways that culminate in apoptosis in this metabolic disorder. ROS levels and apoptosis rates were particularly high in patients P1 and P3, who showed a severe form of CRTR disease and a drastic reduction in the abundance of the *SLC6A8* transcript. It is possible that ROS production exacerbates, helps perpetuate, or causes the neurological phenotype of CRTR patients. Moreover, in patient P4, who had the mildest form of the disease and the highest intracellular Cr content, neither increased ROS nor any other signs of metabolic stress could be detected. However, while ROS production has been reported to possibly affect the nervous system, a complex interplay between GAMT expression and the tumour-suppressor p53 protein has been recently proposed (Ide, et al., 2009). In this new link, GAMT appears as a p53-inducible modulator of apoptosis and a key downstream effector of adaptive response to genotoxic and nutrient stress (Ide, et al., 2009; Vousden, 2010). CDS cells could be used as a model to explore the metabolic functions of p53 and to identify critical points in these pathways that might serve as targets for therapeutics interventions.

In summary, this study is the first in which increased metabolic stress measured as ROS production, apoptosis and/or aberrant cell proliferation has been identified in selected CRTR- and GAMT-deficient fibroblast cell lines (this information is summarised in Supp. Table S3). In addition, a reduction in high energy phosphate reserves appears as a plausible mechanism for explaining the damage observed in selected CRTR fibroblasts. The existence of a dense genetic interaction network makes genotype-phenotype correlations difficult to identify (as for most hereditary diseases) (Dipple and McCabe, 2000a; Dipple and McCabe, 2000b; Lehner, 2007).

However, in the present work, an association was seen between genotypes and metabolic stress phenotypes, especially in certain CRTR patients. The present data support the potential of reducing intracellular ROS and oxidative stress-induced apoptosis as a strategy for the treatment of these CDS.

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FIGURE LEGENDS

Figure 1. Quantification of SLC6A8 and GAMT mRNA by qRT-PCR in CDS patient and control fibroblasts. (A) mRNA level quantification in GAMT patient cell lines. (B) mRNA level

quantification in CRTR patient cell lines. The X axis represents cells according to their type of mutation; the Y axis represents the results of the relative quantification (RQ) of the patient cells, where $RQ=2^{-\Delta\Delta C_T}$. The following formula was used to calculate the relative amount of transcripts in patient cells with respect to controls: $\Delta\Delta C_T = \Delta C_T \text{ patient} - \Delta C_T \text{ control}$. ΔC_T was calculated as the difference in C_T between the target gene and the housekeeping genes for each patient sample. Data represent the mean $\pm$ SD of three independent experiments ($*p<0.05$; $**p<0.01$). P: patient.

Figure 2. Analysis of intracellular ROS content in CDS patient cell lines. Results are expressed as DCF fluorescence relative to the control value. The X axis represents patient cell lines and the Y axis the fluorescence ratio of patient cells with respect to the average of control cell lines (n=6). The data represent the mean $\pm$ SD of three independent experiments each performed in triplicate ($*p<0.05$; $**p<0.01$). P: patient.

Figure 3. Analysis of phosphorylated p38 stress-activated protein kinase expression in patient fibroblasts. Equal amounts of control and patient cell lysates (20 μ g) were loaded and subjected to immunoblotting with the anti-phosphorylated p38 antibody. The stripped blot was retested with total anti-p38 antibody. The bottom panel shows the stripped blot retested with monoclonal anti- α -tubulin antibody (Sigma Aldrich). The results in each case are representative of at least three independent experiments. Patients and their defect group are shown in the upper panel. C: control; P: patient.

Figure 4. Detection of apoptosis in CDS patient and control fibroblasts by flow cytometry. (A) Detection of early apoptotic cells (annexin V-positive and PI-negative cells). (B) Detection of late apoptotic cells (annexin V-positive and PI-positive cells). The X axis represents patient cell lines; the Y axis represents the ratio of annexin V-positive apoptotic cells in each patient with respect to the average for control cells (six lines). The data represent mean $\pm$ SD of three independent experiments each performed in triplicate (* $p<0.05$; ** $p<0.01$). ANX: annexin V; PI: propidium iodide; P: patient.

Figure 5. Analysis of cell cycle distribution in CDS patient and control fibroblasts by flow cytometry. The X axis represents patient cell lines; the Y axis represents the percentage of cells in each stage of the cycle. Data represent mean $\pm$ SD of three independent experiments (* $p<0.05$; ** $p<0.01$). P: patient; C: control.

Table 1. Nucleotide variations in *SLC6A8* and *GAMT*: localisation and prediction of pathogenicity

GENE	EXON	NUCLEOTIDE CHANGE	EFFECT ON PROTEIN	PolyPhen PREDICTION	PROTEIN DOMAIN	CONSERVATION	REFERENCE
<i>SLC6A8</i>	3	c.462G>A	p.W154X				(Fons, et al., 2009)
<i>SLC6A8</i>	6	c.1006_1008delAAC	p.N336del		Cytoplasmic	Highly conserved amino acid in a conserved region for the Na ⁺ /Cl ⁻ -dependent transporter family and orthologous proteins	(Clark, et al., 2006)
<i>SLC6A8</i>	7	c.1079_1081delTCT	p.F360del		TM7	Highly conserved amino acid in a conserved region for the Na ⁺ /Cl ⁻ -dependent transporter family and orthologous proteins	(Sempere, et al., 2009a)
<i>SLC6A8</i>	8	c.1210G>C	p.A404P	Possibly damaging	TM8	Highly conserved region for the Na ⁺ /Cl ⁻ -dependent transporter family and orthologous proteins	(Alcaide, et al. 2010)
<i>SLC6A8</i>	8	c.1222_1224delTTC	p.F408del		TM8	Highly conserved amino acid in a highly conserved region for the Na ⁺ /Cl ⁻ -dependent transporter family and orthologous proteins	(Bizzi, et al., 2002)
<i>SLC6A8</i>	10	c.1396G>A	p.G466R	Probably damaging	TM9	Highly conserved amino acid in conserved region for the Na ⁺ /Cl ⁻ -dependent transporter family and orthologous proteins	Novel
<i>GAMT</i>	1	c.59G>C	p.W20S	Probably damaging Disruption of functional site: <ul style="list-style-type: none"> • Closest contact with ligand SAH • Closest contact with functional site • Cavity creation at buried site 	N-Terminal	Highly conserved amino acid in orthologous proteins	(Item, et al., 2004)
<i>GAMT</i>	1	c.145delT	p.Y49fs				(Sempere, et al., 2009b)
<i>GAMT</i>	2	c.316C>T	p.Q106X				(Sempere, et al., 2009b)
<i>GAMT</i>	4	c.407C>T	p.T136M	Probably damaging <ul style="list-style-type: none"> • Closest contact with ligand SAH • Closest contact with functional site • Closest contact with other chains • Hydrophobicity change at buried site 	Core section	Highly conserved region	(Sempere, et al., 2009b)
<i>GAMT</i>	5	c.521G>A	p.W174X				(Item, et al., 2004)

SAH: S-adenosyl homocysteine. GenBank reference sequences: NM_005629.3 for *SLC6A8*; NM_000156.4 for *GAMT*

Table 2. Intracellular creatine content in fibroblasts from CDS patients and controls

CELL LINE	DEFECT	CREATINE
P1	CRTR	0.60±0.08
P2	CRTR	0.55±0.07
P3	CRTR	0.85±0.35
P4	CRTR	2.30±0.001
P5	CRTR	0.60±0.14
P6	CRTR	0.85±0.07
P7	GAMT	10.0±0.9
P8	GAMT	20.8±4.2
P9	GAMT	NS
Controls (n:9)		15.3±6.8
Range		6.8-31.9

Creatine content expressed as pmol/μg protein
Values are mean ± SD of at least two biological replicas measured in duplicated.
NS: not studied

Figure 1: Quantification of SLC6A8 and GAMT mRNA by qRT-PCR in CDS patient and control fibroblasts. (A) mRNA level quantification in GAMT patient cell lines. (B) mRNA level quantification in CRTR patient cell lines. The X axis represents cells according to their type of mutation; the Y axis represents the results of the relative quantification (RQ) of the patient cells, where $RQ=2^{-\Delta\Delta CT}$. The following formula was used to calculate the relative amount of transcripts in patient cells with respect to controls: $\Delta\Delta CT = \Delta CT \text{ patient} - \Delta CT \text{ control}$. ΔCT was calculated as the difference in CT between the target gene and the housekeeping genes for each patient sample. Data represent the mean $\pm$ SD of three independent experiments (* $p<0.05$; ** $p<0.01$). P: patient.

Figure 2: Analysis of intracellular ROS content in CDS patient cell lines. Results are expressed as DCF fluorescence relative to the control value. The X axis represents patient cell lines and the Y axis the fluorescence ratio of patient cells with respect to the average of control cell lines (n=6). The data represent the mean $\pm$ SD of three independent experiments each performed in triplicate (* p <0.05; ** p <0.01). P: patient.
186x79mm (200 x 200 DPI)

Figure 3: Analysis of phosphorylated p38 stress-activated protein kinase expression in patient fibroblasts. Equal amounts of control and patient cell lysates (20 μ g) were loaded and subjected to immunoblotting with the anti-phosphorylated p38 antibody. The stripped blot was retested with total anti-p38 antibody. The bottom panel shows the stripped blot retested with monoclonal anti- α -tubulin antibody (Sigma Aldrich). The results in each case are representative of at least three independent experiments. Patients and their defect group are shown in the upper panel. C: control; P: patient.
180x113mm (200 x 200 DPI)

Figure 4: Detection of apoptosis in CDS patient and control fibroblasts by flow cytometry. (A) Detection of early apoptotic cells (annexin V-positive and PI-negative cells). (B) Detection of late apoptotic cells (annexin V-positive and PI-positive cells). The X axis represents patient cell lines; the Y axis represents the ratio of annexin V-positive apoptotic cells in each patient with respect to the average for control cells (six lines). The data represent mean $\pm$ SD of three independent experiments each performed in triplicate (* p <0.05; ** p <0.01). ANX: annexin V; PI: propidium iodide; P: patient.

Figure 5: Analysis of cell cycle distribution in CDS patient and control fibroblasts by flow cytometry. The X axis represents patient cell lines; the Y axis represents the percentage of cells in each stage of the cycle. Data represent mean $\pm$ SD of three independent experiments (* $p < 0.05$; ** $p < 0.01$). P: patient; C: control.
280x198mm (200 x 200 DPI)

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Supp. Table S1. Biochemical and genetic characterisation of CRTR patients

PATIENT	DEFECT	GENOTYPE	Cr/Crn NV<1.5	CLINICAL HISTORY
1 ^{F,1}	CRTR	c.462G>A p.W154X	3.2; 4.9	Male 11 y. Psychomotor delay, autistic behaviour and epilepsy. Brain ¹ H-MRS: absence of creatine signal
2 ^F	CRTR	c.1006_1008delAAC p.N336del	4.42; 3.7	Male 3 y. Psychomotor and speech delay, convulsions Brain ¹ H-MRS: absence of creatine signal
3 ^{F, 2}	CRTR	c.1079_1081delTCT p.F360del	3.0; 2.6	Male 40 y. Profound mental retardation, refractory epilepsy, no speech, autistic behaviour, psychomotor delay. One affected brother. Brain ¹ H-MRS: reduced creatine signal
4 ^{F, 3}	CRTR	c.1210G>C p.A404P	2.09-3.28	Male 3 y. Expressive language impairment, mild psychomotor retardation. Brain ¹ H-MRS: reduced creatine signal
5 ^F	CRTR	c.1222_1224delTTC p.F408del	1.9; 2.7	Male 16 y. Mental retardation, convulsions and autism. Brain ¹ H-MRS: absence of creatine signal
6	CRTR	c.1396G>A p.G466R	2.4; 4.1	Male 9 y. Generalised developmental delay, dystonia, no speech, epilepsy. Brain ¹ H-MRS: not done

GenBank reference sequences: NM_005629.3 for *SLC6A8*; NM_000156.4 for *GAMT*
NV: Normal value; y: years of age
F: Genetic analysis of family members performed
¹ (Fons, et al., 2009)
² (Sempere, et al., 2009a)
³ (Alcaide, et al., 2010)

Supp. Table S2. Biochemical and genetic characterisation of GAMT patients

PATIENT	DEFECT	GENOTYPE	GAA (mmol/mol Creat) NV (<11y): 13-139 NV (>12y): 12-100	GAMT ACTIVITY (pmol/h.mg prot) NV:100-195	CLINICAL HISTORY
7 ¹	GAMT	c.[59G>C]+[521G>A] p.[W20S]+[W174X]	228	2.8	Female 23y. Severe mental retardation, refractory epilepsy, autistic behaviour Brain ¹ H-MRS: reduced creatine signal
8 ^{F, 2}	GAMT	c.[145delT]+[145delT] p.[Y49fs]+[Y49fs]	201	3.7	Female 12y. Severe mental retardation, refractory epilepsy, autistic behaviour Brain ¹ H-MRS: reduced creatine signal
9 ^{F, 2}	GAMT	c.[316C>T]+[407C>T] p.[Q106X]+[T136M]	374	4.1	Male 47y. Severe mental retardation, autistic behaviour, seizures Brain ¹ H-MRS: reduced creatine signal

GenBank reference sequences: NM_005629.3 for *SLC6A8*; NM_000156.4 for *GAMT*

NV: Normal value; y: years

F: Genetic analysis of family members performed

¹ (Item, et al., 2004)

² (Sempere, et al., 2009b)

Supp. Table S3. Summary of test results in CDS patient cell lines

Patient	Defect	Genotype	Relative Abundance of mRNA (Fold change)	ROS content (Fold change)	Annexin V positive (Fold change)	Sub G0/G1 cells (%)	Intracel Cr (pmol/μg prot)	phospho-p38 /total p38	Clinical Phenotype
P1	CRTR	c.462G>A p.W154X	<i>SLC6A8</i> 0.07±0.02**	1.54±0.14***	1.3±0.04*	14±2.1***	0.60±0.08	0.9±0.35*	Severe
P2	CRTR	c.1006_1008AAC p.N336del	<i>SLC6A8</i> 1.0±0.1	1.2±0.20	1.1±0.20	1±0.5	0.55±0.07	0.07±0.08	Severe
P3	CRTR	c.1079_1081delTCT p.F360del	<i>SLC6A8</i> 0.5± 0.2*	2.4±0.14***	1.8±0.18**	18±11**	0.85±0.35	1.61±0.47*	Severe
P4	CRTR	c.1210G>C p.A404P	<i>SLC6A8</i> 1.2±0.3	1.1±0.32	0.95±0.28	0.9±0.5	2.30±0.001	0	Mild
P5	CRTR	c.1222_1224delTTC p.F408del	<i>SLC6A8</i> 0.7±0.2	1.3±0.45	1±0.26	0.8±2.7	0.60±0.14	0.39±0.1	Severe
P6	CRTR	c.1396G>A p.G466R	<i>SLC6A8</i> 0,9±0.001	1±0.15	1.1±0.20	0.9±8.6	0.85±0.07	0.10±0.17	Severe
P7	GAMT	c.[59G>C]+[521G>A] p.[W20S]+[W174X]	<i>GAMT</i> 0.3±0.1*	1.9±0.22***	1.5±0.40**	15±0.9***	10±0.9	0.35±0.09	Severe
P8	GAMT	c.[145delT]+[145delT] p.[Y49fs]+[Y49fs]	<i>GAMT</i> 0.06±0.006**	1.12±0.160	0.8±0.06**	0.5±0.4	20.8±4.2	0.01±0.01	Severe
P9	GAMT	c.[316C>T]+[407C>T] p.[Q106X]+[T136M]	<i>GAMT</i> 0.3±0.1*	1.9±0.30**	2±0.56**	18±2.7**	NS	0.07±0.11	Severe
Controls	-	-	1	1	1	0.8± 0.9	15.3 ± 6.8	0.07±0.03	-

GenBank reference sequences: NM_005629.3 for *SLC6A8*; NM_000156.4 for *GAMT*
NS; not studied.
The data represent the mean ± SD of three independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$