Prevalence of Creatine Deficiency Syndromes in Children With Nonsyndromic Autism

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BACKGROUND AND OBJECTIVE: Creatine deficiency may play a role in the neurobiology of autism and may represent a treatable cause of autism. The goal of the study was to ascertain the prevalence of creatine deficiency syndromes (CDSs) in children with autism spectrum disorder (ASD).

METHODS: In a prospective multicenter study, 443 children were investigated after a confirmed diagnosis of ASD. Random spot urine screening for creatine metabolites (creatinine, guanidinoacetate, creatinine, and arginine) with liquid chromatography-tandem mass spectrometry and second-tier testing with high-performance liquid chromatography methodology was followed by recall testing in 24-hour urines and confirmatory testing by Sanger-based DNA sequencing of GAMT, GATM, and SLC6A8 genes. Additional diagnostic tests included plasma creatine metabolites and in vivo brain proton magnetic resonance spectroscopy. The creatine metabolites in spot urine in the autism group were compared with 128 healthy controls controlled for age.

RESULTS: In 443 subjects with ASD investigated for CDS, we had 0 events (event: 0, 95% confidence interval 0–0.0068), therefore with 95% confidence the prevalence of CDS is <7 in 1000 children with ASD. The autism and control groups did not vary in terms of creatine metabolites ($P > .0125$) in urine.

CONCLUSION: Our study revealed a very low prevalence of CDS in children with nonsyndromic ASD and no obvious association between creatine metabolites and autism. Unlike our study population, we expect more frequent CDS among children with severe developmental delay, speech impairment, seizures, and movement disorders in addition to impairments in social communication, restricted interests, and repetitive behaviors.

WHAT’S KNOWN ON THIS SUBJECT: The observation of autistic symptoms in patients with creatine deficiency syndromes implies that impairment in creatine metabolism may play a role in the neurobiology of autism and if so it may represent a treatable cause of autism.

WHAT THIS STUDY ADDS: Our study revealed a very low prevalence of creatine deficiency syndromes in children with nonsyndromic autism spectrum disorder and no obvious association between creatine metabolites and autism.

Autism spectrum disorder (ASD) denotes a range of neurodevelopmental disabilities of complex etiology that can cause significant behavioral, social, and communication challenges. Prevalence of ASD is estimated to be 1 in 68 children (14.7 per 1000) in the United States. Genetic causes (chromosomal abnormalities or genetic alterations) together account for roughly 10% to 20% of ASD cases. The current standard of care genetic test for ASD involves using chromosomal microarrays to assess for pathogenic copy number variations in defined highly penetrant genes or loci. ASD can also occur with inborn errors of metabolism, also known as inherited metabolic disorders, which occur in 1 in 800 live births. Fewer than 5% of ASD cases are attributable to inherited metabolic disorders for which screening is routine, such as phenylketonuria.

The observation of autistic symptoms in patients with creatine deficiency syndromes (CDSs) implies that impairment in creatine metabolism may play a role in the neurobiology of ASD and if so it may represent a treatable cause of ASD. The prevalence of CDS in children with ASD is unknown.

CDSs comprise a group of inborn errors of metabolism that are not routinely screened for during newborn screening, or during standard ASD diagnostic processes. There are 3 known disorders: deficiencies in arginine:glycine amimidotransferase (AGAT-D), guanidinoacetate methyltransferase (GAMT-D), and creatine transporter (CrT-D). Clinical features of CDSs generally include developmental delay/regression, intellectual disability, speech and language deficits, and epileptic seizures; many of which are also evident in children with ASD. Treatment-refractory epilepsy, and extrapyramidal movement disorders present exclusively in GAMT-D, which has a clinical spectrum that encompasses mild and intermediate cases, as well as the most severe phenotype among CDSs that includes severe intellectual deficits. AGAT-D has a milder phenotype, which includes developmental delay, speech impairment, and limited social contact, with mild seizures reported in some cases. Boys with X-linked CrT-D generally have intellectual disability, speech and language deficits, show behavioral abnormalities, and may have seizure disorders, whereas girls usually manifest a continuum from normal to mild involvement. Patients with all forms of CDS have been reported to have ASD symptoms.

Individuals with CDS have dysfunctional creatine metabolism leading to lack of creatine/phosphocreatine in the brain. AGAT-D and GAMT-D are defects in the biosynthesis of creatine and are both inherited as autosomal recessive conditions. The AGAT gene (Gene ID 2628, official nomenclature: GATM) has been mapped to chromosome 15q15.3, is 16.8 kb in size, and contains 9 exons, which encode a protein of 424 amino acids. The GAMT gene (Gene ID 2593, GAMT) has been mapped to chromosome 19p13.3, is 4.46 kb, and contains 6 exons that encode a protein of 237 amino acids. The CrT defect is an X-linked disease of impaired cellular creatine uptake. The CrT gene (Gene ID 6535, SLC6A8) has been mapped to Xq28, spanning 8.4 kb and consisting of 13 exons, which encode a protein of 635 amino acids. To date, there are 6, 50, and 88 pathogenic mutations described throughout GATM, GAMT, and SLC6A8 genes, respectively. A clinical diagnosis of CDS is based on the determination of an abnormal ratio of creatine-to-creatine in spot urine; altered excretion of creatine, guanidinoacetate, and creatinine measured in 24-hour urine collections; altered concentration of these metabolites in blood, or depletion of creatine in brain assessed with in vivo magnetic resonance (MR) spectroscopy. Each of the disorders has a pathognomonic biochemical phenotype that allows the differentiation between them. In GAMT-D, guanidinoacetate is found to be highly elevated and creatine is low; in AGAT-D, guanidinoacetate and creatine both are low; and in CrT-D, the urinary creatine-to-creatinine ratio is elevated.

Creatine deficiency could play a role in the neurobiology of ASD due to its essential function in central nervous system cellular energy homeostasis as a temporal and spatial energy buffer, energy transducer, and general regulator of cellular energetics, as well as action as a neuroprotective agent. In cultured cells, creatine supplementation protects rat hippocampal neurons against glutamate and amyloid β-peptide toxicity. Data using human fetal striatal and mesencephalic tissue identified creatine as a potent natural survival and neuroprotective factor for γ-aminobutyric acid–ergic neurons in a model for Huntington disease and of dopaminergic neurons in a model for Parkinson disease.

Recent interest in routine screening children with ASD for inborn errors of metabolism, and in particular CDS raises the question of how many children with ASD could potentially benefit from such screening. The purpose of the current study is to establish the prevalence of CDS in children with ASD, using a rational approach of biochemical and genetic testing for creatine metabolism.
METHODS

Sample

This prospective study enrolled a cohort of children 2 to 18 years of age between 2010 and 2012 from 3 sites within the Autism Speaks–Autism Treatment Network (ATN)/Autism Intervention Research Network on Physical Health (AIR-P): (1) Holland Bloorview Kids Rehabilitation, Surrey Place Centre, and the Hospital for Sick Children in Toronto, Ontario, Canada; (2) University of Colorado in Denver, CO; and (3) the Lurie Centre for Autism at the Massachusetts General Hospital for Children in Boston, MA.

Participants had a diagnosis of ASD from expert clinicians based on Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria for ASD (Autism, Asperger, or Pervasive Developmental Disorder-not otherwise specified), with the diagnosis confirmed by the Autism Diagnostic Observation Schedule (ADOS-G). The Toronto site recruited an additional group of children with moderate to severe ASD eligible for a publicly funded Intensive Behavioral Intervention therapy program for children with ASD. Children included from this subgroup also underwent assessment by expert clinicians; however, their assessment included observation with the Childhood Autism Rating Scale and the DSM-IV checklist, rather than the ADOS. Children included from this subgroup also underwent assessment by expert clinicians; however, their assessment included observation with the Childhood Autism Rating Scale and the DSM-IV checklist, rather than the ADOS. Children were also included if they had a clinical diagnosis of ASD by ATN expert clinicians by using DSM-IV criteria, without ADOS confirmation. Individuals were excluded if they had Rett syndrome, childhood disintegrative disorder, severe bilateral visual impairment, or severe bilateral hearing impairment.

The study was approved by the research ethics board of The Hospital of Sick Children and the institutional review boards of the other participating ASD ATN/AIR-P Network sites. Subjects enrolled in the study after parents completed written informed consent.

Study Design and Measurement

To diagnose CDS in children with ASD, the study protocol involved 3 main stages: initial screening of random spot urine samples, 24-hour urine samples for those positive in initial screening, and diagnostic confirmation of CDS by gene sequencing for those tested positive in the 24-hour urine. In case of suspicion for AGAT deficiency, we requested, in addition, a plasma sample and when possible and indicated, we performed in vivo [1H]-MR spectroscopy even if AGAT gene sequencing was negative (Fig 1).

In the first stage, high-throughput spot urine testing for creatine and its metabolites; guanidinoacetate, arginine, and creatinine (together with creatine referred to as
creatinine metabolites throughout the text), was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a modified method for urine as described by Tran et al.\(^3\)\(^2\) Interpretation of results was based on age-matched reference ranges (mean ± 2 SD) established with our method from the study cohort. The decision limits were obtained from the analysis of specimens from the first 50 probands and then dynamically adjusted with increasing sample size. Results were calculated as analyte-to-creatinine ratio (mmol/mol creatinine). All results outside the dynamic reference range were flagged “positive.” As second-tier test, samples positive by LC-MS/MS underwent high-performance liquid chromatography (HPLC) guanidino compound analysis by cation-exchange chromatography with postcolumn derivatization.\(^3\)\(^3\) The test was considered positive if HPLC confirmed the presumptive LC-MS/MS positive test, or it was considered negative if HPLC did not confirm.

In the second-stage recall testing, we requested 24-hour urine samples from those subjects in whom spot urine testing was positive in LC-MS/MS and second-tier HPLC tests to achieve higher diagnostic test specificity. For cases with suspected AGAT-D or GAMT-D, the excretion of creatine metabolites was normalized for body weight and collection time and results were expressed as μmol per kg body weight per day. For suspected CrT-D, the creatine-to-creatinine ratio in the collection urine was assessed. For those 24-hour urines with doubt of completeness of the collection, the assessment was made by normalization for creatinine instead of body weight and collection time.

In the third and last stage, confirmation testing consisted of full sequencing of the respective genes in all subjects with abnormal analysis in the 24-hour urine for AGAT-D, GAMT-D, or CrT-D. Sanger-based DNA sequencing chemistry was used (ABI-3730) for polymerase chain reaction–based sequencing of the 3 genes GAMT (AGAT), GAMT, and SLC6A8 (CrT). The coding regions, flanking intronic segments, and ∼500 bp upstream of the genes were sequenced. Examination of raw data was completed manually for missense, nonsense, or small insertion/deletion events.

In addition, in all cases with 24-hour urine results indicating AGAT-D, the creatine metabolites were also analyzed in plasma by applying the HPLC guanidino compound method.\(^3\)\(^3\) In an attempt to achieve highest possible assurance for exclusion of AGAT-D, in the cohort from Toronto, in vivo [\(^1\)H]-MR spectroscopy of the brain was added as the test with the highest diagnostic sensitivity for AGAT-D. Children were sedated and examined using either a 1.5-T or a 3.0-T Philips Achieva clinical MR imaging system (Philips Healthcare, Andover, MA). Two different left-sided localizations starting with basal ganglia and continued with parieto-occipital periventricular white matter with choice of volume according to size of the brain area with focus on tissue homogeneity (minimal voxel size of 1 cm\(^3\)) were investigated by point-resolved spectroscopy acquisitions of each localization (echo time = 144 ms, number of excitations = 128, repetition time = 2000 ms). After normalization for brain water content, the total creatine content (tCR = creatine+phosphocreatine) was quantified using the postanalytical LCmodel software (LCMODEL Inc., Oakville, ON, Canada).\(^3\)\(^4\) Results were referenced to age-matched data from children who underwent [\(^1\)H]-MR spectroscopy investigation for clinical purposes at the Hospital for Sick Children.

The primary outcome sought was the number of subjects with ASD diagnosed with CDS as indicated by creatine metabolites present through both, spot urine and 24-hour-collection urine LC-MS/MS and HPLC assays, as well as confirmation through GAMT, GAMT, or SLC6A8 gene sequencing.

**Controls**

Healthy volunteers ranging in age from 6 months to 18 years from the greater Toronto area were recruited based on defined inclusion and exclusion criteria as described previously.\(^3\)\(^5\)

**Statistical Analysis**

Analysis of covariance adjusting for age was used to examine the association between group and outcomes such as creatinine, creatine, guanidinoacetate, and arginine. Bonferroni correction technique was applied to adjust for multiple testing. The outcomes were positively skewed so square root transformations were performed. Normality of residuals from the models were checked and met. For the calculation of the confidence bounds on the prevalence of CDS the rule of 3 was applied to estimate the upper limit of the probability of an event that has not occurred. The formula for the 1-sided 95% upper confidence bound is 3/\(n\), where \(n\) is the number of probands.\(^3\)\(^6\)

**RESULTS**

A total of 450 subjects enrolled in the study, with 93 from Denver, 134 from Boston, and 223 from Toronto; 443 subjects had spot urine samples analyzed. The mean age at spot urine sampling was 7.3 years (median 6.0, range 2.0–18.7 years). Table 1 presents the demographic and clinical characteristics of the 443 subjects with urine samples. The sample for this study was predominately boys (371, 81%), with a diagnosis of autism (all 443, 100%) by using DSM-IV criteria in conjunction with the ADOS.
From the latter, all 3 had normal tCR in brain measured by [1H]-MR spectroscopy (Fig 2). In 1 of the 9 presumptive AGAT-D cases, plasma was not requested because brain tCR was normal. One of the 4 who tested negative in plasma had also normal tCR.

To rule out ascertainment bias, we surveyed the medical centers for diagnosis and management of patients with inherited metabolic disorders in Toronto, Denver, and Boston. The centers confirmed that there was no new diagnosis made and no new patient seen with AGAT-D, GAMT-D, or CrT-D during the study period.

We observed 0 cases of CDS among 443 children with ASD tested (upper 1-side 95% confidence bound = 0.0068). Therefore, we are 95% confident that the prevalence of CDS is <7 in 1000 among children with ASD.

**DISCUSSION**

This is the first large comprehensive population-based study looking at the prevalence of CDS in ASD in a pan-ethnic group. Despite speculation that creatine deficiency is common in children with ASD, our findings suggest that inborn errors of creatine synthesis (AGAT-D and GAMT-D) and creatine transport (CrT-D) are unlikely to play a role in the pathogenesis of ASD in many children. Two previous investigations into the prevalence of CDS in patients with ASD had similar findings: urine of 203 children with nonsyndromic ASD found none of the children being affected with CDS; screening of 100 boys with ASD for a mutation in the SLC6A8 gene (CrT defect, X-linked) revealed 1 male patient with a novel gene variant falsely reported as “affected.” Of note, this patient had a normal urine creatine/creatinine ratio and normal creatine uptake studies in fibroblasts, thus excluding CrT-D. The same gene variant c.1162G>A was identified in 1 boy in our study and considered as non-disease-causing single nucleotide polymorphism.

**TABLE 1** Demographic and Clinical Characteristics of the Study Sample

<table>
<thead>
<tr>
<th>No. of Subjects</th>
<th>Ethnicity</th>
<th>Gender</th>
<th>TD Score</th>
<th>Age, y</th>
<th>Gender</th>
<th>Enrollment, (n = 443)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toronto</td>
<td>228</td>
<td>Male</td>
<td>81%</td>
<td>7.3</td>
<td>Male</td>
<td>344</td>
</tr>
<tr>
<td>Boston</td>
<td>122</td>
<td>Male</td>
<td>81%</td>
<td>7.3</td>
<td>Male</td>
<td>344</td>
</tr>
<tr>
<td>Denver</td>
<td>93</td>
<td>Male</td>
<td>81%</td>
<td>7.3</td>
<td>Male</td>
<td>344</td>
</tr>
</tbody>
</table>

**ADOS and DSM-IV criteria**

- ATN: 231
- Non-ATN: 123
- CARS and DSM-IV, n (%): 34 (8)
- DSM-IV (expert clinician), n (%): 55 (12)

**Current level of language, n = 437, n (%)**

- Fluent: 196 (45)
- Phrases: 96 (22)
- Single words: 80 (18)
- Nonverbal: 65 (15)

**DQ, n = 344, n (%)**

- No delay: 88 (25)
- Mild delay (DQ 0.67–0.90): 126 (37)
- Moderate delay (DQ 0.33–0.66): 90 (26)
- Severe delay (DQ < 0.33): 40 (12)

**History of seizures, n = 427, n (%)**

- None: 378 (88)
- Febrile seizures: 18 (4)
- One afebrile seizure: 5 (1)
- Seizure disorder well controlled: 25 (6)
- Seizure disorder poorly controlled: 1 (<1)

**ADOS, autism diagnostic observation schedule; ATN, autism treatment network; CARS, childhood autism rating scale.**
The excretion of creatine and guanidinoacetate metabolites in spot urines in our autism group fits well with age-matched "reference data" from other centers using LC-MS/MS. Furthermore, the comparison with healthy children controlled for age in our study revealed no significant differences and is in accordance with a study comparing 57 children with ASD, 49 typically developing siblings, and 49 unrelated controls.

The investigation of collection urine corrects for ~50% of false-positive spot urine tests, which matches our past experience. The metabolite excretion adjusted for body weight proved helpful for the exclusion of AGAT-D and GAMT-D, whereas the collection of urine over a period of time increased the diagnostic specificity for CrT-D by reducing the effect of random creatine intake.

For the exclusion of AGAT-D in cases with positive 24-hour urine, our protocol included additional tests so that we would not miss any affected child who could efficiently be treated by creatine supplementation. The limited body of evidence in AGAT-D suggests that decrease of creatine and especially guanidinoacetate has a higher diagnostic sensitivity and specificity in plasma than in urine.

Also, because even gene sequencing can miss cases, we included the investigation of brain tCR by \(^{1}H\)-MR spectroscopy as the test with highest sensitivity.

The study included higher-functioning children with ASD with no or very mild delays (DQ > 0.9), and who spoke in phrases and sentences. This resulted in a more generalized sample of children with ASD but also had less power to detect the prevalence of CDS in lower-functioning individuals with...
ASD. Although we are not aware that it happened, the requirement of urine sampling may have caused enrollment bias toward toilet-trained subjects who would be higher functioning and/or older. The sample also had a higher mean age, as there was no exclusion criterion for the time since ASD diagnosis. Although age itself would not be a bias in the detection of CDS, the sample may be biased with subjects who continue to seek expert follow-up care, and who have not had an etiological diagnosis after substantial workup. As another limitation of the study, it cannot be excluded that a child with a normal urine profile was affected with CDS because subjects with normal spot urine results were not further analyzed with gene sequencing, plasma testing, or brain [1H]-MR spectroscopy. However, urine analysis represents the standard in clinical testing for CDS and lack of sensitivity has not been observed.

This study did not find any evidence for CDS in a cohort of children with ASD, and even if larger studies could further consolidate the prevalence figures it would, in our opinion, not add much valuable data.

CONCLUSIONS

Without finding any children with ASD positive for CDS in a large sample, inborn errors of creatine metabolism do not seem to represent a contributory cause for ASD. This study is consistent with the previous literature that there is no need to routinely screen children with ASD for CDS. Efforts to look elsewhere for causation of ASD are suggested by this work, and it may be possible that a shared feature might cause the separate but similar ASD and CDS autistic symptoms. However, considering the treatable nature of CDS, children should still be investigated for the possibility of CDS if they present with symptoms such as developmental delay, speech impairment, seizures, and movement disorder in addition to impairments in social communication, restricted interests, and repetitive behaviors.

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ABBREVIATIONS

ADOS: Autism Diagnostic Observation Schedule
AGAT-D: arginine:glycine amidinotransferase deficiency
AIR-P: Autism Intervention Research Network on Physical Health
ASD: autism spectrum disorder
ATN: Autism Treatment Network
CDS: creatine deficiency syndrome
CrT-D: creatine transporter defect
DQ: developmental quotient
DSM-IV: Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition
GAMT-D: guanidinoacetate methyltransferase
HPLC: high-performance liquid chromatography
LC-MS/MS: liquid chromatography-tandem mass spectrometry
MR: magnetic resonance
tCR: total creatine content
FINANCIAL DISCLOSURE: The authors have indicated they have no financial relationships relevant to this article to disclose.

POTENTIAL CONFLICT OF INTEREST: Dr Scherer holds patents for autism biomarkers; the other authors have indicated they have no potential conflicts of interest to disclose.

REFERENCES


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