

Double-Blind Therapeutic Trial in Angelman Syndrome Using Betaine and Folic Acid

Sarika U. Peters,^{1,2} Lynne M. Bird,³ Virginia Kimonis,⁴ Daniel G. Glaze,¹ Lina M. Shinawi,¹ Terry Jo Bichell,² Rene Barbieri-Welge,³ Mark Nespeca,⁵ Irina Anselm,⁶ Susan Waisbren,⁶ Erica Sanborn,⁶ Qin Sun,¹ William E. O'Brien,¹ Arthur L. Beaudet,¹ and Carlos A. Bacino^{1*}

¹Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas

²Vanderbilt Kennedy Center for Research on Human Development, Nashville, Tennessee

³Department of Pediatrics, University of California, San Diego, California

⁴University of California, Irvine, California

⁵Department of Neurosciences, University of California, San Diego, California

⁶Children's Hospital Boston, Boston, Massachusetts

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Angelman syndrome (AS) is caused by reduced or absent expression of the maternally inherited ubiquitin protein ligase 3A gene (*UBE3A*), which maps to chromosome 15q11–q13. *UBE3A* is subject to genomic imprinting in neurons in most regions of the brain. Expression of *UBE3A* from the maternal chromosome is essential to prevent AS, because the paternally inherited gene is not expressed, probably mediated by antisense *UBE3A* RNA. We hypothesized that increasing methylation might reduce expression of the antisense *UBE3A* RNA, thereby increasing *UBE3A* expression from the paternal gene and ameliorating the clinical phenotype. We conducted a trial using two dietary supplements, betaine and folic acid to promote global levels of methylation and attempt to activate the paternally inherited *UBE3A* gene. We performed a number of investigations at regular intervals including general clinical and developmental evaluations, biochemical determinations on blood and urine, and electroencephalographic studies. We report herein the data on 48 children with AS who were enrolled in a double-blind placebo-controlled protocol using betaine and folic acid for 1 year. There were no statistically significant changes between treated and untreated children; however, in a small subset of patients we observed some positive trends. © 2010 Wiley-Liss, Inc.

Key words: Angelman syndrome; methylation; high dose folic acid; betaine; imprinting disorders; treatment; promotion of methylation

INTRODUCTION AND BACKGROUND

The clinical phenotype of Angelman syndrome (AS) includes global developmental delay, minimal or absent speech, seizures, ataxia, sleep disturbance, and an unique behavioral profile consisting of happy demeanor, easily provoked or inappropriate laughter, hypermotoric behaviors, and excessive mouthing behaviors [Williams

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and Frias, 1982; Zori et al., 1992; Williams et al., 1995a,b]. Treatment for this condition is supportive and mainly consists of controlling seizures and employing rehabilitative therapies.

AS is caused by several recognized mechanisms, which reduce the expression of *UBE3A*: (1) 5–6 Mb deletions of the maternal 15q11–q13 region (65–75%) [Wagstaff et al., 1992; Christian et al., 1995]; (2) paternal uniparental disomy (pUPD) with two copies of paternally inherited 15q11–q13 and no copy of maternally inherited 15q11–q13 (3–5%) [Knoll et al., 1991; Nicholls et al., 1992; Freeman et al., 1993]; (3) imprinting defects where the

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*Correspondence to:

Carlos A. Bacino, M.D., Texas Children's Hospital, 6701 Fannin St., Clinical Care Center Suite 1560, Houston, TX 77030.

E-mail: cbacino@bcm.tmc.edu

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maternally inherited *UBE3A* has the methylation and gene expression pattern of a paternally inherited *UBE3A*, thereby rendering the maternally inherited gene inactive (6–8%) [Horsthemke 1997; Horsthemke et al., 1997; Buiting et al., 1998]; and (4) loss-of-function point mutations or intragenic deletions in the maternally inherited *UBE3A* gene (4–6%) [Kishino et al., 1997; Matsuura et al., 1997; Jiang et al., 1999]. Haploinsufficiency for genes adjacent to *UBE3A* may contribute to a more severe phenotype in deletion cases. There remains a group of patients with AS with no identifiable molecular abnormality (10–14%).

UBE3A is subject to genomic imprinting [Nakao and Sasaki, 1996] and expression of *UBE3A* is essential to prevent AS. *UBE3A* is expressed from both paternal and maternal chromosomes in most tissues, but in neurons in most regions of the mouse brain, only the maternal gene is expressed [Kashiwagi et al., 2003; Dindot et al., 2008]; the situation in humans is presumed to be analogous. Purkinje cells of the cerebellum and regions of the hippocampus demonstrate almost exclusively maternal expression of *UBE3A* [Rougeulle et al., 1997; Vu and Hoffman, 1997; Jiang et al., 1998a,b]. If the maternal copy of *UBE3A* is deleted, inactive, or mutated, AS results [Stalker and Williams 1998; Jiang et al., 1999].

Protocol Rationale and Hypothesis

This study was based on the hypothesis that increased methyl donors in the diet may increase DNA methylation, increase expression of the paternally inherited *UBE3A* gene, thereby activating the silenced allele and ameliorating the symptoms of AS. In order to test this hypothesis, we administered high-dose folic acid and betaine in a double-blind placebo-controlled fashion to children with AS for 1 year. Clinical effects were measured with biochemical markers, electroencephalographic (EEG) recordings, clinical, and neurodevelopmental evaluations.

Although the function of the *UBE3A* gene has not been completely elucidated, it is regulated by an imprinting center (AS-IC) in the 15q11–q13 region, adjacent to the *SNRPN* gene. The promoter for *SNRPN* overlaps with the PWS portion of the imprinting center (PWS-IC) and is fully methylated on the maternally inherited chromosome and completely unmethylated on the paternally inherited chromosome. Rougeulle et al. [1998] identified a brain-specific antisense transcript and hypothesized that it plays a role in silencing of the paternal *UBE3A* allele in the brain. When a region of the IC in the paternal *UBE3A* gene is hypomethylated, a long transcript is expressed near the *SNRPN* gene and extending to the antisense strand [Runte et al., 2001] and when the antisense is transcribed, the *UBE3A* gene is apparently silenced. There is a tissue-specific region of differential methylation (TS-DMR) near the 3'-end of the *UBE3A* gene that is fully methylated in liver and lymphoblasts but predominantly unmethylated in cerebellum and hippocampus, and variably methylated in different sub regions of cerebral cortex [Jiang et al., 2004]. The available data are consistent with the hypothesis that antisense for *UBE3A* and silencing of the maternal allele occur only when both the PWS-IC and the TS-DMR are unmethylated as on the paternal allele in cerebellum and hippocampus. Since this region on the paternal chromosome is fully unmethylated, and associated with expression of the *UBE3A* antisense (*UBE3A-ATS*) transcript; we hypothesized

that methylation of the 3'-CpG in the *UBE3A* (TS-DMR) and the AS-IC on the paternal chromosome gene could lead to a decrease of *UBE3A-ATS* transcript and further expression of the paternal *UBE3A* allele and rescue the phenotype caused by the maternal *UBE3A* deficiency.

Altering gene expression by altering methylation has been shown to be a viable strategy in mice [Wolff et al., 1998; Cooney et al., 2002]. Feeding pregnant mice a methyl-donor-supplemented diet alters the coat color of the mouse pups. Differences in expression of the agouti alleles are associated with changes in DNA methylation, indicating that both methylation and gene expression can be influenced by dietary intake in mice [Cooney et al., 2002]. Due to epigenetic regulation, differential expression of the coat color is seen in genetically identical mice. In fact, failure to suppress the agouti allele epigenetically during development causes overexpression of this gene later in life. The agouti overexpression in mice gives rise to the yellow agouti obese mouse syndrome secondary to a number of downstream metabolic and endocrine abnormalities [Wolff et al., 1998; Cooney et al., 2002]. The differences in expression of the agouti alleles are therefore associated with changes in DNA methylation and highlights the effects of gene expression by modifying dietary intake [Cooney et al., 2002].

In humans, moderate folate depletion decreases DNA methylation in blood lymphocytes in postmenopausal women, and this hypomethylation can be reversed with folic acid administration (286–616 µg daily) [Jacob et al., 1998]. There is also a report that hyperhomocystinemia seen with renal failure can result in inappropriate biallelic expression of imprinted genes and that the abnormality can be corrected by administration of folic acid [Ingrosso et al., 2003]. Folic acid is the synthetic form of the naturally occurring folate, and it has been used in relatively high doses in pregnant women to prevent recurrence of neural tube defects, with no known harmful side effects [Willett, 1992; Daly et al., 1995; Sayers et al., 1997; Felkner et al., 2005; Quinlivan and Gregory, 2007]. Children with metabolic disorders affecting folate metabolism have been treated with doses of up to 20 mg/day without adverse effects [Holme et al., 1989; Kishi et al., 1994]. Betaine is a naturally occurring metabolite of choline. When given in high doses to patients with methylenetetrahydrofolate reductase (MTHFR) deficiency, cobalamin cofactor defects, and cystathionine B-synthase deficiency, it increases plasma levels of methionine [Wendel and Bremer, 1984; Holme et al., 1989; Sakura et al., 1998]. Large increases in plasma methionine are not associated with adverse clinical consequences or the development of tolerance, even after many years of treatment [Walter et al., 1998].

AS patients who have UPD have a slightly milder phenotype than deletion patients [Freeman et al., 1993; Saitoh et al., 1997]. The presumed explanation for this observation is that the silencing of the paternally inherited *UBE3A* allele is incomplete, and having two copies of an incompletely silenced allele provides slightly more *UBE3A* expression and mitigates the phenotype somewhat. The incomplete silencing of the paternally inherited *UBE3A* might be exploited by a mechanism that further impairs silencing, thereby increasing *UBE3A* expression from the paternally inherited allele.

Arn et al. [1998] described a patient with MTHFR deficiency who presented a clinical phenotype of AS. Since MTHFR deficiency impairs production of methyl donors for the methylation of DNA,

one explanation for the AS-like phenotype in this patient is impaired methylation of the maternally inherited *UBE3A* allele. In other words, the primary metabolic defect in this patient may have caused the AS phenotype by affecting methylation status and *UBE3A* gene expression. This suggests that if methylation status of the paternally inherited *UBE3A* allele could be altered, gene activation could occur and effect a treatment for AS.

METHODS

Description of Subjects

The enrollment target was 60, although the final accrual was 57 patients, 19 average in each site. All patients had a molecularly confirmed diagnosis of AS. Due to attrition, only 48 patients finished the study and were used for the analysis. Participants ranged in age between 5 months and 14 years. Of the 48 participants, there were 16 females and 32 males; 37 had deletions, 6 had uniparental disomy, 2 had imprinting center defects, and 3 had *UBE3A* mutations. Three sites were involved in this study: Baylor College of Medicine, Children's Hospital Boston, and Rady Children's Hospital, San Diego. The Institutional Review Boards at all the participating institutions approved this protocol.

Patients with AS were excluded from the study if they had one of the following: (1) A clinical diagnosis of AS with no identifiable molecular genetic abnormality; (2) previous drug, vitamin, or dietary manipulations related to folic acid or betaine; (3) severe, uncontrolled seizures or other problem rendering the patient medically unstable; or (4) pernicious anemia.

Dosages and Randomization

The treatment trial was a 12-month double-blind, placebo-controlled trial with participants randomized to the folate–betaine combination or to the placebo group. The Investigational Pharmacy at one of the three study sites randomized the participants. There were 20 numbers initially assigned to each site. The medications were randomized at one of the sites and ultimately dispensed by the local Pharmacies involved in the study. All medications were re-packaged in identical containers to conceal the identity of the treatment provided. All the professionals including investigators, nurses, and study coordinators were unaware of the selection until the code was broken at study completion. Investigators and parents proved unable to surmise which patients were on drug versus placebo.

The dose of folic acid was 15 mg orally per day, administered as a single daily dose. The dose of betaine was 6 g orally per day divided into 2 g three times daily for patients weighing <30 kg and 12 g orally per day for patients weighing 30 kg divided into 3 g four times daily.

To maintain proper nutritional balance, all subjects were given an over-the-counter pediatric multivitamin per day. Each multivitamin contained 400 µg of folic acid and the dose recommended was 1/2 tablet (200 mcg) for children <13 kg, and a full tablet (400 mcg) for children >13 kg.

Evaluations

Anthropometry, physical and neurological examinations, developmental assessments and EEG studies were performed at baseline and 12 months. EEG studies with 16–18 channels were performed

on subjects with a full complement of electrodes placed using the standard 10/20 system. Recordings of 40–60 min in both wake and sleep states were attempted without sedation. All studies were reviewed by board certified neurophysiologists.

Developmental assessments were conducted by child psychologists using standardized instruments. The instruments applied were (1) Bayley Scales of Infant Development, Second Edition (Mental Scale; BSID-II) [Bayley, 1993] which provide psychomotor and mental developmental indices and are accurate to a developmental age of 42 months; (2) Vineland Adaptive Behavior Scales—Inter-Interview Edition (VABS) [Sparrow et al., 1984], a structured parent interview assessing daily living skills, communication, socialization, and motor skills; and (3) Preschool Language Scales-3, which assess auditory comprehension and expressive communication in children up to 6 years 11 months of age.

Many children with AS exhibit features that overlap with those of autism. This issue has been studied by other researchers in the past as well as by our group [Steffenburg et al., 1996; Peters et al., 2004]. Because of that, formal evaluations for autism were obtained during the study in a selected group of patients. Those children were given the Autism Diagnostic Observation Schedule Generic, Module 1 (ADOS-G). All of the parents of children with AS who exceeded cut-offs for autism or autism spectrum diagnoses on the ADOS were also given the Autism Diagnostic Interview—Revised (ADI-R).

In addition to the formal developmental testing, parent questionnaires to record subjective impressions of change in development and behavior and to survey for side effects, were completed every 2 months by in-person or telephone interview. The parent/caretaker was asked to indicate if the AS patient has experienced worsening, no change or improvement in each of the following: (1) sleep; (2) hyperactivity; (3) drooling; (4) feeding; (5) walking; (6) hand use; (7) alertness; (8) communication; (9) mood; and (10) other. Other areas assessed in the questionnaire included: oral sensitivity and texture aversions, toileting abilities, tremors, balance while sitting or standing, non-verbal communication, presence of hypermotoric behavior, and attention span (see supporting information which may be found in the online version of this article).

Laboratory investigation included a complete blood count (CBC), red blood cell (RBC) folate contents, blood urea nitrogen (BUN), creatinine, and urine analysis at baseline, 6 months and 12 months, performed at various local clinical laboratories. Because some patients did not return to the study sites for the 6-month evaluation, laboratory data was not collected at 6 months on all 48 patients and therefore only lab values taken at baseline and 12 months are reported. Plasma levels of homocysteine (Hcy), methionine, betaine (trimethylglycine), dimethylglycine (DMG, a by-product of betaine), creatine, and guanidinoacetate (GAA) were analyzed at baseline and 12 months at a central facility using a Quattro Micro tandem mass spectrometer (Waters Corporation, Milford, Massachusetts). Creatine and GAA were measured to monitor the potential shuttling of methyl groups to the synthesis of creatine, normally driven by the conversion of GAA to creatine. Quantitative determinations were made using selected mass transitions with the addition of appropriate stable isotopes when possible. The results were not known to the investigators or study coordinators until completion of the study in order to maintain blinded status.

Statistical Design

The primary outcome variables were: (1) differences in biochemical measurements; (2) onset of seizures onset or number of seizure medications used; (3) the difference in scores achieved on formal developmental assessments at baseline and at the completion of 1 year of treatment; (4) differences in parental reports from questionnaires. One-way ANOVA's or Chi-square analyses were used to test for differences between treatment and placebo groups. For one-way ANOVA's, age was used as a covariate. Statistical significance was defined as $p < 0.05$.

RESULTS

Although the recruitment initially aimed for 60 participants, several participants were lost to follow-up. Fifty-one participants completed all developmental evaluations and a total of 48 participants had biochemical testing done in blood at baseline and 12 months. Twenty patients from Baylor College of Medicine, 20 patients from Rady Children's Hospital, San Diego, and 8 patients from Boston Children's Hospital completed the study. Thirty-eight children had a complete assessment for autism. Only participants with complete medical, developmental and biochemical data were included in this analysis.

Biochemical Analysis

Table I shows the mean values for biochemical analyses at baseline and after 12 months of supplementation, comparing patients on folate and betaine (treatment) versus placebo. There were no statistically significant differences observed between the treatment and placebo groups at baseline for any of these biochemical parameters. After 1 year of supplementation, the treatment group had significantly higher betaine and DMG levels as expected. The normal values for the biochemical analytes studied were as follows: creatine 30–120 $\mu\text{mol/L}$, GAA 0.4–4.0 $\mu\text{mol/L}$, methionine 9–45 $\mu\text{mol/L}$, total plasma Hcy 4–14 $\mu\text{mol/L}$, RBC folate 280–903 ng/ml. No standardized values for normal ranges exist for betaine and DMG (values expressed in micromoles per liter).

There were no statistically significant differences observed at baseline between the medication and placebo groups prior to the treatment for any of the measurements performed.

There was a statistical significance for betaine, DMG, and RBC folate values in treated individuals compared to placebo at the $p = 0.05$ level when comparing the baseline to the 12 months

values. The creatine, Hcy, methionine, and GAA levels trended downwards although with no significant differences found at the $P = 0.05$ level. The measurements for betaine and DMG can be used as indicators for compliance that was judged to be adequate. We did not detect any significant changes in the other laboratory parameters (CBC, urine analysis, BUN, and creatinine) that were done to monitor for toxicity (results not shown).

Seizures

Since most AS patients develop seizures by age 3, and high doses of folic acid have been implicated in exacerbation of seizures; we monitored all participants for onset of seizures, as well as seizures control for those affected. The age of onset of seizures for the placebo group was 27.6 ± 14.4 months. The age of onset of seizures for the treatment group was 34.2 ± 20.1 months. These were not significantly different ($F = 1.53, P = 0.22$).

The number of anticonvulsants being taken was used as a surrogate measure for severity of seizures (Table II). No significant differences were observed in the number of anticonvulsants reported by subjects in the treatment group compared to the placebo group. These findings suggest that treatment with folic acid and betaine did not trigger new seizures or worsen existing seizures ($X^2 = \text{NS}$).

Developmental Evaluations

The results for the BSID-II and the PLS-3 developmental assessments are shown in Table III. Results are displayed as the mean difference in raw scores achieved at baseline and 12 months. Age was used as a covariate in all analyses. There were no significant differences in these developmental parameters between the placebo and treatment groups. There were no significant differences in parental responses to the VABS between the placebo and the treatment groups (data not shown).

Prior studies indicate that children with Angelman Syndrome tend to plateau in their development once they attain developmental ages of 24–30 months [Williams et al., 2006]. We conducted an analysis comparing the responses of younger versus older children to treatment to explore whether younger children were more likely to have a response to medications. This analysis was done by examining the correlation between age and the difference in scores from baseline and the results are summarized in Table IV. When examining differences in primary outcome according to age,

TABLE I. Biochemical Parameters at Baseline and After 12 months of Supplements (n = 48 at 12 Months)

		Betaine ($\mu\text{mol/L}$)	DMG ($\mu\text{mol/L}$)	RBC folate (ng/ml)	Creatine ($\mu\text{mol/L}$)	Meth ($\mu\text{mol/L}$)	Hcy ($\mu\text{mol/L}$)	GAA ($\mu\text{mol/L}$)
Placebo group	Baseline (n = 29)	51.45	9.26	614.45	58.43	50.83	5.37	1.202
	12 months (n = 28)	79.52	12.96	746.17	49.49	20.13	3.74	1.045
Treatment group	Baseline (n = 24)	58.41	10.74	638.54	71.36	58.41	6.40	1.148
	12 months (n = 20)	599.69 ^a	64.72 ^a	894.95 ^a	47.73	29.02	3.45	1.296

^aStatistically significant difference from placebo at $p < 0.05$.

TABLE II. Number of Seizure Medications

	Number of anticonvulsants taken				
	0	1	2	3	4
Treatment (n)	3	9	6	1	1
Placebo (n)	5	15	7	1	0

the results indicated that parents of younger children on treatment were more likely to report improvements in the socialization skills domain of the VABS, as compared with parents of younger children on placebo ($P < 0.03$). In other words, for children in the treatment group, younger age correlated with higher rates of improvement in socialization skills according to parent report. Improvements were noted in motor skills (BSID-II and VABS) for younger children, whether or not they were on treatment. In other words, regardless of being on medication or placebo, younger children had greater improvements in motor skills than older children.

Autism

In performing our standard developmental evaluations, we have observed that many of the patients with Angelman Syndrome displayed a number of autistic behaviors. This observation prompted us to include a formal evaluation for autism using ADOS-G and ADI-R. There was a suggestion that individuals with AS and co-morbid autism made less progress in some areas of development on treatment than participants with AS and autism who received placebo. There was a trend toward improvement in some developmental parameters in non-autistic individuals with AS on treatment compared with those who received placebo. No statistical significance could be demonstrated ($P = NS$) ultimately because of the small numbers when participants were classified by age, treatment status, and autism status. Participants with AS without autism made substantially more progress than those with AS and autism, regardless of treatment/placebo status.

Questionnaire Data

Differences between medication and placebo groups were examined according to parental responses to the questionnaire (see supporting information for questionnaire items which may be found in the online version of this article). Results revealed that the only statistically significant difference observed for any ques-

tionnaire items was that parents of children on medication reported lower levels of hypermotoric behavior after 12 months on treatment as compared to children on placebo ($F = 2.2$; $P = 0.02$). More specifically, both parents of children on medications as well as placebo reported that their children were better able to stay still and calm for a longer duration, but parents of children on medications reported more improvements in their child's ability to do this as compared to scores from baseline (refer to supporting information, section S for specific scale which may be found in the online version of this article).

DISCUSSION

AS is a neurodevelopmental disorder with severe mental impairments, neurological deficits, seizures, sleep disturbances, and lack of speech among its major clinical manifestations. Because of the unique biology of *UBE3A*, we hypothesized that dietary manipulation could be used to modify gene expression. We have used the dietary supplements folic acid and betaine in an attempt to modify methylation and influence gene expression, with the hope of ameliorating the phenotype.

No difference in any developmental parameter was found between AS individuals taking high doses of folic acid and betaine and those given placebo. Additionally, no differences were noted on most parental report measures. We did note that younger children on medications seemed to have more improvements in socialization (according to parental report on the VABS). Additionally, we noted that parents of children on medications reported greater improvements in hypermotoric behavior (i.e., their children were able to stay still and calm for a longer period of time) as compared to children given placebo.

Children who were on medications and did not have a diagnosis of co-morbid autism, showed an upward trend in their development but differences were not statistically significant. Participants without autism made more progress than those with a co-morbid diagnosis of autism, regardless of treatment. When stratified by both autism and treatment status, the subgroups of patients were small, limiting the power to show statistical significance. There was a non-significant trend for children with co-morbid autism who received treatment to make fewer gains in development than children with co-morbid autism on placebo.

We found an increase in levels of betaine and DMG, a by-product of betaine, in the plasma samples of patients on treatment, likely as a result of treatment compliance with the treatment regimen. We also detected an increase in the concentration of RBC folate in both groups, which was expected in those receiving high doses of folic acid, but was also seen in placebo subjects. This may be due to the

TABLE III. Mean Difference in Raw Scores Achieved at Baseline and End of Treatment

	PLS-3			BSID-II	
	Receptive language	Expressive language	Total language	Mental development	Motor development
Treatment (n = 20)	1.80	1.27	2.70	8.79	7.07
Placebo (n = 28)	1.42	1.13	2.67	9.05	6.50

TABLE IV. Correlations Between Age at Baseline and Improvement in Scores Over Time For Medication and Placebo Groups

	BSID-II		VABS			
	Cognition	Motor skills	Communication	Daily living skills	Socialization	Motor skills
Placebo	-0.16	-0.39 ^a	-0.18	0.27	-0.21	-0.50 ^a
Treatment	-0.34	-0.40 ^a	-0.27	0.06	-0.46 ^a	-0.51 ^a

^a $P < 0.05$.

fact that both groups received a pediatric multivitamin containing folate. Methionine levels were slightly decreased in the treated group. Betaine acts as a methyl-donor for the re-methylation of Hcy to methionine, a step that is facilitated by betaine homocysteine methyl-transferase. This reaction results in elevations of methionine [Yaghmai et al., 2002]. The reason for these lower trend values in our samples is not clear, although these were not statistically significant. The levels of Hcy were slightly lower but not statistically significant. Methionine can be shuttled to form S-adenosyl methionine (SAM) and S-adenosyl homocysteine (SAH) resulting in lower Hcy values. With regards to the creatine levels, they were slightly higher in the treated group although not statistically significant. It can be hypothesized that an increase in SAM in the presence of methyl groups may have driven the reaction to synthesize creatine by the conversion of GAA to creatine [Stead et al., 2004]; however, the trend was modest. No toxicity was evident as per laboratory analyses from the betaine and high dose folate treatment.

This study reports the first attempt at treating AS and had some similarities with a recent study done with Rett syndrome patients [Glaze et al., 2009]. The Rett study was a double-blind protocol done on a group of 68 patients with Rett syndrome using betaine and folic acid, at similar high doses used by our group. The goal of the study was to increase the degree of methylation of some CpG sites, and promote transcriptional repression either of mutant methyl-CpG-binding protein 2 (MECP2) expressed on all tissues but more abundant in the brain, or by other methyl-binding proteins; in order to ameliorate the clinical features of Rett syndrome. Metabolic investigations, clinical evaluations, EEG's, and parental questionnaires were obtained in these patients but no statistically significant improvements were objectively seen although there was some subjective improvement with regards to sleep, hand stereotypies, communication, alertness, and mood according to parental questionnaire results. No adverse reactions were observed from the treatment [Glaze et al., 2009].

In summary, the results of this trial do not warrant the use of folic acid and/or betaine as a treatment for AS. Many parents elected to continue their children on partial treatment beyond the scope of this trial, and those participants continued on 5 mg of folic acid daily. We have not had any documented reports of adverse effects, nor have we received reports of significant improvements in cognition, language, motor skills, or behaviors associated with the continuation of folic acid.

Based on the suggestive trends for improvement in some areas of development, a second trial using a (theoretically) more potent pro-

methylation strategy (betaine, creatine, metafolin, and vitamin B₁₂) was conducted. The rationale is that first, some patients might accumulate unmetabolized folic acid, and the use of metafolin would overcome this issue. Second, we added creatine since normally a large fraction of S-adenosylmethionine is used to synthesize creatine. By supplying creatine, more S-adenosylmethionine might be available for methylation of DNA, and perhaps histones. Data analysis from this clinical trial is in progress, and results of this study will be reported elsewhere. No striking improvements were evident from clinical observations.

To date, two Angelman syndrome mouse models have been created [Jiang et al., 1998b; Miura et al., 2002]. One carries a null mutation of the maternal *UBE3A* gene and is associated with motor dysfunction, inducible seizures, and long-term potentiation (LTP) deficits [Jiang et al., 1998b]. Another mouse model was achieved through a targeted deletion of the maternal *UBE3A* gene [Miura et al., 2002]. This mouse similarly has motor deficits, learning defects, abnormal hippocampal EEG's, as well as defects in context-dependent fear conditioning. In summary, these mice recapitulate many of the clinical findings seen in patients with Angelman syndrome. Given the availability of these animal models, it would be valuable to perform further studies looking at brain specific as well as global methylation patterns, using similar diet manipulations, or other drugs that can alter methylation. It could be argued that in time, more refined tools might become available to detect potential benefit, or that more targeted interventions are needed to alter the specific areas where the gene is imprinted such as hippocampus and cerebellum. We hope that in future studies good surrogate markers may be available to determine the levels of methylation and *UBE3A* activity in specific areas of the brain since global methylation may not necessarily reflect the methylation within specific brain sites. We believe that changing methylation patterns by drug interventions, and perhaps even by gene therapy, remain as a valuable therapeutic approach for future treatments in Angelman syndrome and other disorders subject to genomic imprinting.

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