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ORIGINAL PAPER

An Examination of Changes in Urinary Metabolites and Behaviors with the Use of Leucovorin Calcium in Children with Autism Spectrum Disorder (ASD)

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Abstract

Objectives Children with autism spectrum disorder (ASD) have been found to have a high prevalence of folate receptor auto-antibodies (FRAA), which may impair the normal transport of folate from blood into the cerebrospinal fluid (CSF). Leucovorin calcium (LC) is believed to bypass the folate transport system and restore function. We sought to examine changes in behavior and urinary metabolites in children and young adults with ASD being treated with LC.

Methods Students attending a K-12 school for ASD were recruited for an open-label, 12-week study of high-dose LC (2 mg/kg/day, max dose 50 mg/day). The primary outcome measures were the mean changes in the Aberrant Behavior Checklist (ABC) and the Social Responsiveness Scale (SRS). We also examined changes in Pediatric Quality of Life (PedsQL) and urinary metabolites. Changes were assessed with paired sample *t*-tests.

Results Twelve students aged 13 to 19 (2 girls, 10 boys) completed the study. The parent-reported SRS showed a non-significant decrease (improvement) of 7.8 points (95% CI − 1.6 to 17.3, $p = 0.095$), and the ABC showed a non-significant decrease of 2.4 points (95% CI − 6.4 to 11.3, $p = 0.56$). The teacher-reported ABC and the parent-reported PedsQL showed very little change. Urinary metabolites with the greatest changes were involved in folate, phosphatidylcholine, and tocopherol metabolism.

Conclusions In an open-label study of school-aged children with ASD, LC treatment did not lead to significant improvements. The parent-reported SRS showed a non-significant improvement of 7.8 points, which is clinically important and worthy of future study with larger samples. The potential benefits of LC may be limited to children with a specific physiological abnormality (e.g., FRAA status) and may require a targeted treatment approach. Urinary metabolites may be a useful tool to identify children who are likely to respond to treatment.

Keywords Autism · Leucovorin calcium · Metabolomics · Folinic acid

Prior studies have linked abnormalities in the metabolism of folate, an essential B vitamin, to autism spectrum disorder (ASD) (Vahabzadeh and McDougle 2013). Children with ASD have been found to have impaired transport of folate across the blood-brain barrier due to auto-antibodies that either block or bind to the folate receptor alpha (FR alpha). The antibodies are known as folate receptor auto-antibodies (FRAA). This creates the condition known as "cerebral folate deficiency," which describes an individual with normal serum folate concentrations but low concentrations of folate in the cerebrospinal fluid (CSF) due to impaired ability to transport folate across the blood-brain barrier (Frye et al. 2018). Folate is believed to have many essential functions in the central nervous system. Folate is a component of tetrahydrofolate, which is a cofactor involved in DNA methylation and neurotransmitter synthesis and degradation. Low folate levels in the CSF have been implicated in neural tube defects, neurodegenerative diseases, neuropsychiatric diseases, and cancer (Stover et al. 2017).

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Children with ASD have been found to have a high prevalence of FRAA. In a study of 93 children with ASD, 60% had blocking FRAA, and 44% had binding FRAA (Frye et al. 2013). Blocking antibodies interfere with binding of folate to the FR alpha, and binding antibodies bind to the FR alpha and cause an antibody-mediated immune reaction. Both antibodies interfere with the normal transport of folate into the CSF. The rates of FRAA in ASD are higher than the 4–15% prevalence reported in healthy adults and the 3% prevalence reported in developmentally delayed non-autistic children (Frye et al. 2018). Up to 23% of children with ASD who underwent lumbar puncture were reported to have abnormally low CSF folate concentrations (Shoffner et al. 2016). Together, this evidence suggests that low CSF folate levels may contribute to the abnormal physiology and clinical symptoms of ASD.

Leucovorin calcium (LC—the drug name for folinic acid), a reduced form of folate, is able to bypass the normal blood-brain barrier transport mechanism and increase CSF folate levels through a secondary transport mechanism. Case reports have found that high-dose LC supplementation markedly improves CSF folate levels in children with ASD and low CSF folate (Frye et al. 2018). In a recent randomized controlled trial of LC supplementation vs. placebo in 48 children with ASD and language delay, children randomized to LC had statistically significant improvements in language and aberrant behavior (Frye et al. 2018). Furthermore, improvements were greater in the subgroup of children who were positive for FRAA, suggesting that children with more severe cerebral folate deficiency were more likely to benefit.

Since folate is involved in DNA synthesis, cellular methylation, and redox homeostasis, and an adequate cerebral supply of folate may allow for improved functioning of these pathways and clinical improvements. In order to further examine the efficacy of LC in children with ASD, we conducted an open-label study to determine if treatment would lead to clinical improvements and whether there would be associated changes in urinary metabolites, which might suggest a mechanism of action and help identify children most likely to benefit.

Methods

Participants

Parents and/or caregivers of children and young adults between the ages of 5 and 22 who were enrolled at the Oak Hill School (OHS) in San Anselmo, CA (a school for children with autism or related neurodevelopmental disorders), were recruited by e-mail to participate in the study. Participants were required to be enrolled at OHS, between the ages 5 and 22 and with a diagnosis of ASD (as established by expert clinician review of DSM-V criteria and meeting a threshold score on the Social Communication Questionnaire). Children were excluded if they had a co-existing condition that, in the opinion of the investigator, would compromise the safety of the patient or the quality of the data or if they had a known allergy to any component of the LC product or if they were currently taking LC or had used this supplement in the last 6 months.

Eighteen children were enrolled in the study at initiation; six children dropped out of the study, and twelve children completed the study. Reasons for drop-outs were as follows: (1) increased depression, (1) more easily upset, (1) emotionally sensitive, (1) hyperactivity, (1) diarrhea, and (1) migraine. None of the adverse effects that led to drop-outs were judged to be likely due to treatment. Table 1 shows the characteristics of the children who completed the study ($n = 12$). Most students were male (83.3%) and between the ages of 13 and 17 (75.0%).

Procedure

Minor students and adult students who were under legal conservatorship went through an informed consent process and provided verbal assent, while their parents or guardians provided informed consent. Young adults (age 18 and over) who were not conserved by their parents provided their own consent. Students were excluded if they were not interested/willing to participate in the program or the student or parents/caregivers were not willing to fill out study surveys. Study participants were provided with LC capsules and advised to take them twice a day with meals for 12 weeks. The capsules are dye-free, milk product-free, vegetarian, and available in 3 strengths (5, 10, and 25 mg) and were provided with instructions for weight-based dosing (2 mg/kg with maximum dose of 50 mg/day divided in two doses). The capsules were produced by Lee Silsby Pharmacy (Cleveland Heights, OH). A certificate of analysis was obtained by an independent analytical service, and an investigational new drug packet was approved by the FDA. Participants were advised to swallow the capsules with water or juice, or, if this was not possible, they were advised to pierce the capsule and mix the contents into soft food including yogurt, oatmeal, and drinks and then consume the mixed material.

Measures

All outcome measures were assessed monthly, and the primary outcomes of interest were change in mean scores from baseline to close-out (12 weeks).

The Aberrant Behavior Checklist (ABC), community version, is a 58-item rating scale that measures aberrant behavior in children and young adults with neurodevelopmental disorders and it is a commonly used outcome measure in clinical

Table 1 Baseline characteristics

Characteristics	Number	Percentage
Gender		
Male	10	83.3%
Female	2	16.7%
Age group (years)		
13–17	9	75.0%
≥ 18	3	25.0%
Mean age (SD)	16.3 (1.5)	
Race		
Caucasian	9	75.0%
More than one race	3	25.0%
Medical comorbidities present		
Yes		
Expressive language disorder	2	16.7%
Mixed receptive-expressive language disorder	1	8.3%
Speech and Language Impairment	1	8.3%
Other	2	16.7%
No or not reported	8	66.7%
Current medication use		
Yes		
Anticonvulsant medications	1	8.3%
Anti-depressant medications	5	41.7%
Anti-psychotic medications	3	25.0%
GI medications	1	8.3%
Sedative medications	2	16.7%
Stimulants	2	16.7%
Other medications	2	16.7%
No or not reported	3	25.0%
Supplement use		
Yes	4	33.3%
No or not reported	8	66.7%

trials of interventions for ASD (Bent et al. 2014; Hardan et al. 2012). Studies examining the factor structure of the ABC and comparisons to other scales have demonstrated convergent and divergent validity of the scale (Kaat et al. 2014).

The Social Responsiveness Scale (SRS) is a 65-item rating scale that assesses the severity of social impairment in individuals with ASD. The scale has been shown to have both good sensitivity and specificity be able to distinguish ASD from other childhood psychiatric conditions (Duku et al. 2013). The SRS is commonly used to assess social reciprocity in studies of interventions for ASD (Bent et al. 2014; Hardan et al. 2012) and has high inter-rater reliability between parents (Pearl et al. 2013).

The Pediatric Quality of Life (PedsQL) scale measures overall quality of life and has been used in hundreds of studies of children and young adults with medical and psychiatric disease. PedsQL has demonstrated high reliability and construct validity in healthy, acute, and chronic disease pediatric populations. It is responsive to change and is therefore appropriate for use in clinical trials (Fitzpatrick et al. 2020).

Urinary metabolites were measured for all participants at screening and close-out (0 and 12 weeks). Parents were instructed to assist the study participant with a home urine collection in a sterile container provided by the study team (fasting, 8 a.m. target collection time) and then immediately place the urine sample in their home freezer. A member of the study team picked up the urine sample on the same morning; it was collected and transported in a cooler with ice to the laboratory, where it was placed in a – 80 °C freezer, generally within 1–2 h of collection. Metabolomic assessment was conducted by Metabolon (Research Triangle, NC), and urinary metabolites were normed per gram of creatinine in the urine sample. Metabolon is one of the largest and most established laboratories conducting state-of-the art metabolomics assessments of urine, plasma, and other clinical samples (www.metabolon.com).

Table 2 Changes in mean total scores in each of the outcome measures over time

Outcome measure	Caregiver			Teacher		
	Mean change	95% CI	*p* value	Mean change	95% CI	p value
Aberrant Behavior Checklist	− 2.4	− 11.3 to 6.4	0.56	1.2	− 5.2 to 7.6	0.68
Social Responsiveness Scale	− 7.8	− 17.3 to 1.6	0.095	− 1.7	− 10.56 to 7.16	0.95*
Pediatric Quality of Life	− 0.8	− 5.2 to 3.5	0.69			

$p < 0.05$ indicates statistical significance. *The p value for SRS teacher total scores was analyzed by using paired Wilcoxon test since the difference between paired subjects for SRS teacher total scores were not normally distributed using Shapiro-Wilk test. The Pediatric Quality of Life was completed only by the caregivers and not the teachers

Safety was assessed monthly by asking parents and caregivers whether their child had experienced any adverse medical events or potential side effects while taking LC treatment. Parents/caregivers were also instructed to contact the study team immediately if the participant had any new medical problem that might represent an adverse event. Reported adverse events were summarized in a tabular format.

Data Analyses

We examined the trajectory of change in the mean score of ABC, SRS, and PedsQL at baseline and week 12 using paired sample *t*-test. Differences between paired subjects for SRS teacher total scores were not normally distributed using Shapiro-Wilk test, so we used a paired two-sample Wilcoxon test for SRS teacher total scores. We also measured urinary metabolites at baseline and week 12 to examine their fold of change. All the analysis were conducted using R Studio Version 1.1.456. Because this study is exploratory in nature, no adjustments were made for multiple comparisons, as recommended by Rothman (Rothman 1990).

We conducted a power analysis to determine the ability of this small study to detect a change in social responsiveness. We defined a large change in social responsiveness (SRS) as an effect size of 0.8 (which corresponds to the minimal clinically important difference of 10) and used the standard deviation of the change of the SRS of 12 (from our prior studies). With our sample size of 12 and an alpha of 0.05, the power of this study to detect this specified level of change was 50%, which is below the standard of 80% often used for larger clinical trials, but expected for a small, early intervention study.

Results

Table 2 shows the change in mean difference in all outcome measures before (baseline) and after the children took LC (week 12). None of the observed changes were statistically significant. The parent-reported mean of the differences in SRS showed the biggest change of a non-significant decrease (improvement) of 7.8 points (95% CI − 1.6 to 17.3, $p = 0.095$). The parent-reported ABC showed a non-significant decrease (improvement) of 2.4 points (95% CI − 6.4 to 11.3, $p = 0.56$), and the teacher-reported ABC showed a non-significant increase (worsening) of 1.2 points (95% CI -5.2 to 7.6, $p = 0.68$). The parent-reported PedsQL showed a non-significant decrease (worsening) of 0.8 points (95% CI − 3.5 to 5.2, $p = 0.69$).

As shown in Table 3, the urinary metabolites with the largest change over the course of the study (and their associated fold change) were as follows: 5-methyltetrahydrofolate (24.8), 1-stearoyl-2-arachidonoyl-GPC (10.1), 1-stearoyl-2-oleoyl-GPC (9.3), alpha-tocopherol (9.2), and 1-stearoyl-2-linoleoyl-GPC (7.8).

Adverse events were uncommon in participants who completed the study. Five adverse events were reported over the 12-week period, and none were judged to be serious or likely to be caused by the LC (1 each of upset stomach, dry mouth, cold, increased aggression, and blood in stool).

Discussion

In this open-label study in an unselected, small sample of school-aged children with ASD who were treated with a 12-week course of LC, we did not find statistically significant improvements in behavior, social responsiveness, or quality of life. Interestingly, there was a non-significant improvement in social responsiveness as assessed by parents (7.8 points, effect size = 0.52) which is a medium effect size. While a minimal clinically important difference (MCID) for the SRS in children with autism has not been determined in children with ASD, some authors have suggested using a change in the SRS of $\geq$ 10 to indicate a MCID and to address the concern that smaller changes may be within the range of a placebo effect (Hardan et al. 2019). The non-significant change of

Table 3 Selected urinary metabolites with statistically significant changes from 0 to 12 weeks

Category	Sub-pathway	Biochemical name	Fold of change	*p* Value
Folate metabolism	Folate metabolism	5-Methyltetrahydrofolate	24.8	0.03
Lipid	Phosphatidylcholine	1-Stearoyl-2-arachidonoyl-glycerophosphocholine	10.1	> 0.01
Lipid	Phosphatidylcholine	1-Stearoyl-2-oleoyl-glycerophosphocholine	9.3	0.04
Lipid	Tocopherol metabolism	Alpha-tocopherol	9.2	> 0.01
Lipid	Phosphatidylcholine	1-Stearoyl-2-linoleoyl-glycerophosphocholine	7.8	> 0.01
Neurotransmitter	Acetylated peptides	3-Hydroxyphenylacetoyl-glutamine	0.5	0.02
Neurotransmitter	Glutamate	Carboxymethyl-GABA	1.6	> 0.01
Neurotransmitter	Glutamate	Gamma-carboxyglutamate	1.6	0.02
Neurotransmitter	Tyrosine	Dopamine	1.4	> 0.01
Redox indicator	Methionine	Methionine sulfoxide	1.6	0.02
Redox indicator	Methionine	Cystathionine	1.5	0.048
Redox indicator	Methionine	Cystine s-sulfate	1.4	0.01
Redox indicator	Methionine	N-acetylcysteine	0.6	0.048
Redox indicator	Methionine	Alpha-ketobutyrate	0.4	0.01

Fold changes > 1.0 indicate an increase, and < 1.0 indicate a decrease in the urinary metabolite from 0 to 12 weeks. GABA indicates gamma aminobutyric acid

7.8 points observed in this study is below this proposed MCID threshold but still a signal of a large enough change to warrant further investigation in larger, placebo-controlled studies. As previously mentioned, one prior randomized controlled trial of LC treatment for 12 weeks in children with ASD found statistically significant improvements in behavior and language compared with children treated with placebo (Frye et al. 2018). In that study, children who were positive for the FRAA-autoantibody showed greater improvements than those without the antibody, suggesting that LC may be most beneficial in this subset of children. Our study did not include measures of the FRAA antibody, but selection of subjects who are positive for this antibody may increase the likelihood of observing beneficial effects.

This study examined changes in urinary metabolites during treatment with LC. We found that the urinary metabolites with the largest increases during treatment were related to folate metabolism, phosphatidylcholine, and tocopherol. Although our study had insufficient power to assess the predictive value of these metabolites, larger samples may be able to target these biomarkers to determine if patients with certain urinary profiles are more likely to benefit from treatment.

Limitations and Future Research Direction

Our study was limited by the small sample size in a heterogeneous school population and lack of a control group. The finding of a non-significant improvement in social responsiveness with a medium effect size is intriguing and suggests that a larger study with greater power might find an important therapeutic benefit to LC. It is also possible that a beneficial effect would be larger if the study were limited to participants who are FRAA positive. Although a prior study of LC treatment in children with autism found benefits mostly in speech and communication (Frye et al. 2018), we sought to determine if changes would be found in more global measures of social interaction (SRS), behavior (ABC), and overall quality of life (PedsQL) in a population of school children without requiring subjects to have a specific language impairment. Of note, both the SRS and ABC include questions related to communication and speech. Six subjects dropped out of the study for various reasons, and there were a small number of minor adverse events. The frequency of most of the reasons cited for dropping out is high in this population, so it is not clear if LC contributed to these negative symptoms. Our clinical judgment is that the treatment was generally well tolerated but we advise clinical monitoring in any child who elects to try LC. Future studies might also consider a more gradual increase in does to avoid any possible dose-related side effects.

In summary, there is an interesting physiological rationale to suggest that LC treatment may be beneficial in children with ASD, particularly those who are FRAA positive. Although we did not find a statistically significant benefit, our study was limited by a small sample size, and the magnitude of the observed change is still consistent with a clinically important beneficial effect. We recommend that a large, randomized, placebo-controlled trial be conducted, ideally in participants who are screened for FRAA antibody status. Urinary metabolites in a larger sample hold promise for both observing a mechanism of action and predicting a subset of patients who may be more likely to benefit from treatment.

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Authors' Contributions All authors contributed to the study conception and design. Clinical oversight and review of eligibility of all participants was performed by Robert L. Hendren. Material preparation and data collection were performed by Felicia Widjaja, Yingtong Chen, Jessica Wahlberg, and Michael G. McDonald. Data analysis was performed by Yingtong Chen and Stephen Bent. The first draft of the manuscript was written by Stephen Bent, and all authors commented on previous version of the manuscript. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Conflict of Interest Robert L. Hendren reports receiving research grants in the past year from Curemark, Roche, and Otsuka. He is also on Advisory Boards for BioMarin, Axial Therapeutics, and Janssen. He has conducted reviews and grants for Autism Speaks, the Simons Foundation, and Brain Canada. He receives royalties from Oxford, Routledge, and American Psychiatric Associating Publishing. He is not part of any Speakers Bureaus. All other authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee (University of California, San Francisco Committee on Human Research reference number 17-22860) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed Consent All participants went through an informed consent process that was approved by the University of California, San Francisco Committee on Human Research, prior to participating in any study-related activities.

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