Protocol: Micronutrient supplementation in children with autism spectrum disorder (ASD): A clinical trial examining mechanism of action

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Background:

Prior studies have suggested that children with developmental disorders including autism spectrum disorder (ASD) have nutritional deficiencies and/or imbalances in vitamins and minerals, including low levels of vitamin D, lithium, zinc, and methionine and high levels of vitamin B6, vitamin C, and beta-carotene, among others.[1] These nutritional deficiencies may be responsible for impaired methylation, decreased glutathione levels, and increased oxidative stress seen in children with ASD.[1]

The high prevalence of nutritional deficiencies in ASD may be due to dietary patterns in this population, and multiple studies indicate that children with ASD consume less varied diets with limited fruits and vegetables.[2] Also, children with ASD have a high prevalence of gastrointestinal symptoms including diarrhea, constipation, and abdominal pain,[3] which likely affects the absorption of nutrients and the composition of healthy bacteria in the intestinal tract (microbiome).

There are likely functional consequences to these highly prevalent nutritional deficiencies. One analysis found that autism severity scores were correlated with levels of vitamins, minerals, and amino acids.[1] A double-blind, randomized, placebo-controlled trial of a vitamin/mineral supplement in 141 children with autism found numerous improvements in nutritional biomarkers as well as a statistically significant improvement in parent ratings of child functioning (with specific questions on language, behavior, social function, sleep, gastrointestinal symptoms, and eye contact). The parent rating scale ranged from 1 to 7 (lower scores better), and the children in the treatment group had a 0.34 greater improvement in this score (which was described as a modest benefit). There was no improvement in the Social Responsiveness Scale (SRS) or the Pervasive Developmental Disorder Behavior Inventory (PDD-BI).[4] A subsequent study from the same group evaluated a more complex intervention that included a nutritional supplement as well as digestive enzymes, carnitine, essential fatty acids, Epsom-salt baths, and a healthy gluten-free, casein-free, soy free diet. The study found improvements in autism symptoms but was limited by the single-blind study design.[5]

Micronutrient supplementation in other developmental disorders, including Attention Deficit/Hyperactivity Disorder (ADHD), have also shown clinical benefits. In a randomized controlled trial of 135 children (mean age 9.8) with ADHD, 54% of children treated with micronutrients were defined as

"responders" on a clinical global impression scale vs. only 18% of children in the placebo group. The treated children also had a small but significantly greater increase in height (6mm), but the parent ratings were not improved in the treatment group.[6]

Several potential mechanisms of action of micronutrients supplementation on improved clinical outcomes have been proposed, including changes in gut microbiota, methylation capacity, and inflammation.[7-9] Further clarification of the mechanism of action of micronutrient supplementation has the potential to identify specific pathways involved in clinical benefits and allow for more targeted treatment by screening children for nutritional deficiencies and modifying supplement formulations to optimize benefits. We previously demonstrated the value of using urinary metabolomics to identify physiological changes associated with clinical outcomes in a clinical trial of sulforaphane (an antioxidant supplement) in children with ASD.[10] Fifteen children were enrolled and had a comprehensive urinary metabolomic analysis performed at baseline and after 8 weeks of treatment with sulforaphane. Five metabolic pathways – oxidative stress, amino acid/gut microbiome, neurotransmitters, hormones, and sphingomyelin metabolites were correlated with changes in clinical outcome measures. The sphingomyelin metabolite identification was novel and suggested a possible new line of research to determine whether antioxidant supplementation might alter this important component of neuronal development.[10]

Since micronutrient deficiencies are common in children with ASD, and because there is scientific evidence to suggest that supplementation may produce important clinical benefits, we seek to more precisely examine the mechanism of action of micronutrient supplementation through a clinical trial using comprehensive, before-and-after metabolomic analyses. Since the prior study, the metabolomics field has advanced so that it is now possible to obtain metabolomic analyses using Dried Blood Spots (DBS), which are minimally invasive (requiring only a fingerstick and a drop of blood), and provide a much broader metabolomic analysis than urine. We therefore plan to use this new technology in the current study. The analyses may suggest specific metabolic changes that underlie clinical improvements in ASD, which may provide a greater understanding of the physiology of ASD and lead to improved, targeted treatment options.

Methods:

Approvals: The clinical trial will be reviewed and approved by the UCSF Committee on Human Research prior to performing any study activities. The study protocol will be registered at clinicaltrials.gov.

Participants: The UCSF investigative team has an ongoing relationship with a local, non-public school (Oak Hill School, San Anselmo, CA) that specializes in the education of children and young adults with autism and related neurodevelopmental disorders (ages 5-22, grades K-12). This unique academic-school-parent partnership was created with the goal of improving overall care and communication between caregivers, clinical providers, and teachers. All children/families attending the school (n=50 currently) will be invited to participate in the study through e-mail, informational flyers, and an evening informational session.

Children and young adults will be eligible to participate if they are enrolled in the school, have a formal diagnosis of autism, report no use of micronutrient supplements within the last 3 months, are willing to hold other treatments constant for the 8-week study period, have no major medical problems other than ASD, are willing to provide urine samples, and parents were willing to complete on-line surveys at specified intervals. ASD is defined as being present if the child has a diagnosis from a medical professional trained to diagnose autism, or if the student is determined by school staff and the study psychiatrist to meet *Diagnostic and Statistical Manual of Mental Disorders*, Fourth Edition (*DSM–IV*) criteria for ASD. Informed consent will be obtained from the parent/caregiver of all child study participants and directly from all adult study participants. Adults who are conserved will go through an informed consent process and provide assent, and their conservator will be asked to provide informed consent.

Intervention:

The planned study is open-label and all clinicians, parents, and teachers will be aware of the treatment initiation and duration of 8 weeks. All enrolled children will be provided a micronutrient supplementation (EM Power Plus).

Objectives and outcomes:

The primary goal of the study is to determine if any observed changes in symptoms of ASD are correlated with changes in metabolites that occur during treatment with micronutrients. We hypothesize that micronutrient treatment will lead to changes in physiological pathways and that those changes will correlate with clinical improvements.

The two primary outcome measure is the correlation of the changes in clinical symptoms with the change in specific metabolites. Two measures will be used to assess the change in core and associated features of autism: the Autism Behavior Inventory, Short Form (ABI-S) and the Social Responsiveness Scale (SRS). The ABI-S is an observer-reported outcome scale designed specifically to measure change and severity of ASD symptoms and has been shown to have content validity when completed by caregivers.[11] The SRS is one of the most commonly used outcome measures in clinical trials of ASD and focuses on social responsiveness and interaction.[12] Parents/caregivers will be asked to complete both measures at baseline, 4 weeks, and 8 weeks using an on-line and secure platform.

Dried blood spots (DBS) will be collected on the day of study initiation and on the final day of the study after the 8-week treatment period. We will be using "batch enrollment" where the parents and caregivers of all potentially eligible participants will be screened for eligibility and asked to go through an informed consent process. Once the final "set" of eligible student participants is identified, they will all be given the study medication and have the DBS sample collected on the same day. DBS is collected using a simple, sterile, finger-prick device and technique, which generates a drop of blood. The participant will be assisted to place his/her finger on a special DBS "card" which will collect the drop of blood. DBS cards will be individually packaged and stored immediately at -80 °C. During the screening visit, children will have a brief physical examination including height and weight to guide the proper dosing. Micronutrient treatment will be provided at the screening visit along with dosing instructions, but participants will not begin the treatment until the study initiation day, when the DBS samples are collected.

Safety assessments:

Parents/caregivers and teachers will be advised to report any concerns about a new medical problem immediately to the study investigators, who will be available at all times to receive reports of possible adverse effects. At the 4 week and 8-week on-line questionnaires, parents/caregivers will be asked to report any new medical problems or concerns for possible side effects.

Metabolomic analyses:

All DBS samples will be sent on dry ice in one batch to Metabolon (Morrisville, NC). Metabolomics analyses will be conducted at Metabolon as previously described [13]. Briefly, samples will be subjected to methanol extraction, then split into five aliquots for analysis by ultrahigh performance/mass spectrometry in the positive (two methods), negative or polar ion modes. Metabolites will be identified

by automated comparison of ion features to a reference library of chemical standards followed by visual inspection for quality control [14]. For statistical analyses and data display, any missing values will be assumed to be below the limits of detection; these values will be imputed with the compound minimum (minimum value imputation). Data will then be normalized by measured osmolality, which is necessary to reduce the variability [15]. A metabolic pathway for a given metabolite will be assigned based on prior designations in the literature combined with experience from prior datasets at Metabolon.

Statistical methods:

Summary statistics will be used to describe the variables. Change in both the clinical variables and the metabolites will be computed as post-test minus pre-test. Pearson's correlation coefficient will be estimated and tested to index the association between the change in each of the two clinical scales and the change in each metabolite. Given the early-stage nature of the research, we will not adjust for multiple comparisons because we believe it was more important to risk a Type II error than to miss a potentially important signal by being overly conservative, as has been suggested by prior authors [16].

We define, *a priori*, that a correlation cutoff of an absolute value of $\geq$ 0.6 is of potential clinical relevance, because, given our planned sample size of 25, we will have approximately 80% power to detect a correlation of that size and such a correlation accounts for roughly one-third of the variance. Others have suggested that correlations with an absolute value of $\geq$ 0.6 indicate a moderate or higher correlation [17] (and are hence of greatest interest in pointing to a mechanism of action).

We also plan to examine the number of participants who have a "clinical response," defined *a priori* as an improvement of 4 or more points in the ABI-S. We will compare the pre-post changes in the ABI-S and SRS in the responder and non-responder groups using the student's t-test.

Planned Data Tables:

Table 1: Characteristics of Enrolled Participants

Category	**Characteristic**	**Subjects N=15**	
		%	**N**
Gender	Male		
	Female		
Ethnicity	White		
	Asian/Pacific Islander		
	No response		
Age	7-10		
	11-14		
	15-21		
	Mean Age		
Primary diagnosis	Autism Spectrum Disorder		
Comorbidities	Intellectual Disability		
	Language Disorder		
	ADHD		
	Pica		
	Global Development Delay		
	Learning Disability		
	Other		
Current meds	Sertraline		
	Lurasidone		
	Risperidone		
	Birth Control		
	Zonisamide		
	Other		

Table 2: Metabolite correlations

	Primary Outcome Measure				
Metabolite	**ABI-S**		**SRS**		**Metabolic Pathway**
	Corr.	**p-value**	**Corr.**	**p-value**	
Amino acids (endogenous)					
Benzene metabolism					
Cholesterol metabolism					
Fatty acids					
Monoterpene phenol					
Neurotransmitters					
Oxidative stress					
Polyol					
Sphingomyelin					
Sugars					
TCA cycle					
Other					

Table 3. Change in outcome scores over the 12-week study period

Outcome Measure	Adjusted Mean Scores (95% CI)			Change from Baseline (95% CI)			
	Baseline	**1 month**	**3 months**	**1 month**	**p**	**3 months**	**p**
Autism Behavior Inventory Total Score							
Social Communication							
Restrictive and Repetitive Behaviors							
Mental Health							
Self-regulation							
Challenging Behaviors							
Social Responsiveness Scale Total Score							
Awareness							
Cognition							
Communication							
Mannerisms							
Motivation							

Mean change scores will be adjusted for age and gender; *indicates statistically significant change from baseline

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