UNIVERSITY OF CALIFORNIA, SAN DIEGO

SAN DIEGO STATE UNIVERSITY

# Choline Intervention in Children with Fetal Alcohol Spectrum Disorders

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Clinical Psychology

by

Tanya T. Nguyen

Committee in charge:

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Professor Sarah N. Mattson
Professor Edward P. Riley

2015

The Dissertation of Tanya T. Nguyen is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

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2015

# DEDICATION

For my parents, Thu and Neville Alexander. Thank you for your constant love and unconditional support throughout this journey. Your steadfast patience, encouragement, and confidence in me are unparalleled, and I truly would not have been able to make it without you.

To my dearest friends and JDP classmates and colleagues. Your friendship, support, and empathy have made this process and the ultimate product of my dissertation more fulfilling and enriching. I am incredibly grateful and fortunate to have experienced this journey through graduate school with all of you.

# EPIGRAPH

"I don't like work—no man does—but I like what is in the work—the chance to find yourself. Your own reality—for yourself, not for others—what no other man can ever know. They can only see the mere show, and never can tell what it really means."

~ Joseph Conrad, *Heart of Darkness*

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ACKNOWLEDGEMENTS

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Chapters I-IV, in part, are currently being prepared for submission for publication of the material. Nguyen, T. T.; Risbud, R. D.; Chambers, C.; & Thomas, J. D. The dissertation author was the primary investigator and author of this material.

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## PUBLICATIONS

*Peer-reviewed articles*

Infante, M. A., Moore, E. M., **Nguyen, T. T**., Fourligas, N., Mattson, S. N., & Riley, E. P. (2014). Objective assessment of ADHD core symptoms in children with heavy prenatal alcohol exposure. *Physiology & Behavior*. Advanced online publication. doi: 10.1016/j.physbeh.2014.10.014

**Nguyen, T. T.**, Glass, L., Coles, C. D., Kable, J. A., May, P. A., Kalberg, W. O., Sowell, E. R., Jones, K. L., Riley, E. P., Mattson, S. N., & the CIFASD. (2014). The clinical utility and specificity of parent reports of executive functions among children with prenatal alcohol exposure. *Journal of the International Neuropsychological Society*, 20, 704-716.

**Nguyen, T. T.**, Thomas, J. D., Ashrafi, A., Riley, E. P., & Simmons, R. S. (2013). Children with heavy prenatal alcohol exposure have different frequency domain signal characteristics when producing isometric force. *Neurotoxicology & Teratology*, *35*, 14-20.

**Nguyen, T. T.**, Levy, S. S., Riley, E. P., Thomas, J. D., & Simmons, R. W. (2013). Children with heavy prenatal alcohol exposure experience reduced control of isotonic force. *Alcoholism: Clinical and Experimental Research*, *37*, 315-324.

Simmons, R. W., **Nguyen, T. T.**, Levy, S. S., Thomas, J. D., Mattson, S. N., & Riley, E. P. (2012). Children with heavy prenatal alcohol exposure exhibit deficits when regulating isometric force. *Alcoholism: Clinical and Experimental Research*, *36*, 302-309.

Mattson, S. N., Crocker, N., & **Nguyen, T. T.** (2011). Fetal alcohol spectrum disorders: Neuropsychological and behavioral effects. *Neuropsychology Review*, *21*, 81-101.

*Book chapters*

**Nguyen, T. T.**, & Riley, E. P. (2014). The effects of prenatal alcohol exposure on brain and behavior. In B. Carpenter, C. Blackburn, & J. Egerton (Eds.), *Fetal Alcohol Spectrum Disorders: Interdisciplinary perspectives* (pp. 219-240). London, UK: Routledge.

**Nguyen, T. T.**, & Thomas, J. D. (2011). Fetal alcohol spectrum disorders and nutrition. In R. E. Tremblay, M. Boivin, R. V. Peters, & R. G. Barr (Eds.), *Encyclopedia on Early Childhood Development* (pp. 1-8). Montreal, Quebec: Centre of Excellence for Early Childhood Development.

**Nguyen, T. T.**, Coppens, J., & Riley, E. P. (2010). Prenatal alcohol exposure, FAS, and FASD: An introduction. In E. P. Riley et al. (Eds.), *Fetal Alcohol Spectrum Disorders: Management and policy perspectives of FASD* (pp. 1-15). Weinheim, Germany: Wiley-Blackwell.

*Manuscripts currently under review*

Simmons, R. W., **Nguyen, T. T.**, Thomas, J. D., & Riley, E. P. (under review) The use of open- and closed-loop control during goal-directed force responses by children with heavy prenatal alcohol exposure.

Ashrafi, A, **Nguyen, T. T.**, Thomas, J. D., Riley, E. P, & Simmons, R. W. (under review). Classification of children with fetal alcohol spectrum disorders utilizing principal component analysis of force signals.

*Manuscripts in preparation*

**Nguyen, T. T.**, Keen, C. L., Chambers, C. D., & Thomas, J. D. Effects of prenatal alcohol exposure on choline metabolism: A translational approach. Manuscript in preparation.

## PEER-REVIEWED PRESENTATIONS

**Nguyen, T. T.**, Risbud, R., Chambers, C., & Thomas, J. D. (2014, June). Dietary nutrient intake is associated with hyperactivity among children with prenatal alcohol exposure. Paper and poster presented at the annual meeting of the Research Society on Alcoholism, Bellevue, WA. **Selected as a finalist for the Enoch Gordis Research Recognition Award*

**Nguyen, T. T.**, Glass, L., Coles, C. D., Kable, J. A., May, P. A., Kalberg, W. O., Sowell, E. R., Jones, K. L., Riley, E. P., Mattson, S. N., & the CIFASD. (2013, October). The clinical utility and specificity of parent reports of executive functions among children with prenatal alcohol exposure. Paper presented at the annual conference of the National Academy of Neuropsychology, San Diego, CA. **Awarded the NAN Student Poster Award*

**Nguyen, T. T.**, Glass, L., Coles, C. D., Kable, J. A., May, P. A., Kalberg, W. O., Sowell, E. R., Jones, K. L., Riley, E. P., Mattson, S. N., & the CIFASD. (2013, June). Clinical utility of the Behavioral Rating Inventory of Executive Function in the identification of children with prenatal alcohol exposure. Poster presented at the annual meeting of the Research Society on Alcoholism, Orlando, FL.

Infante, M. A., Moore, E. M., **Nguyen, T. T**., Bazinet, A. D., Mattson, S. N., & Riley, E. P. (2013, June). Objective assessment of ADHD core symptoms in children with heavy prenatal alcohol exposure. Poster presented at the annual meeting of the Research Society on Alcoholism, Orlando, FL.

**Nguyen, T. T.**, Thomas, J. D., Riley, E. P., & Simmons, R. W. (2013, February). Goal-directed regulation of force in children with heavy prenatal alcohol exposure. Poster presented at the annual meeting of the International Neuropsychological Society, Waikoloa, HI.

**Nguyen, T. T.**, Simmons, R. W., Mattson, S. N., Sowell, E. R., Thomas, J. D., & Riley, E. P. (2012, June). Neural correlates of force regulation in children with heavy prenatal alcohol exposure. Poster presented at the annual meeting of the Research Society on Alcoholism, San Francisco, CA.

**Nguyen, T. T.**, Keen, C., Chambers, C. D., & Thomas, J. D. (2012, June). The effects of prenatal alcohol exposure on choline metabolism: Implications for how choline may moderate alcohol's teratogenic effects. Paper presented at the Children's Environmental Health Network Research Conference: The Contribution of Epigenetics in Pediatric Environmental Health, San Francisco, CA.

**Nguyen, T. T.**, Simmons, R. W., Levy, S. S., Mattson, S. N., Thomas, J. D., & Riley, E. P. (2011, September). Children with heavy prenatal alcohol exposure experience reduced control of isotonic force. Paper presented at the NIAAA Training Directors Meeting and Trainee Workshop, Providence, RI.

**Nguyen, T. T.**, Keen, C., Chambers, C. D., & Thomas, J. D. (2011, June). The effects of prenatal alcohol exposure on choline metabolism: Implications for how choline may moderate alcohol's teratogenic effects. Paper presented at the annual meeting of the Research Society on Alcoholism, Atlanta, GA.

**Nguyen, T.T.**, Keen, C., Chambers, C.D., & Thomas, J.D. (2011, March). The effects of prenatal alcohol exposure on choline metabolism: Implications for how choline may moderate alcohol's teratogenic effects. Paper presented at the annual SDSU Student Research Symposium, San Diego, CA.

**Nguyen, T. T.**, Simmons, R. W., Levy, S. S., Thomas, J. D., Riley, E. P., & Mattson, S. N. (2010, November). Regulation of isotonic force in children with and without heavy prenatal alcohol exposure. Poster presented at the annual meeting of the Society for Neuroscience, San Diego, CA.

Simmons, R. W., Ashrafi, A., **Nguyen T. T.**, Levy, S. S., Thomas, J. D., & Riley, E. P. (2010, November). Frequency response characteristics of isometric force regulation in children with prenatal alcohol exposure. Poster presented at the annual meeting of the Society for Neuroscience, San Diego, CA.

Simmons, R. W., **Nguyen, T. T.**, Levy, S. S., Thomas, J. D., & Riley, E. P. (2010, June). Regulation of sustained isometric force in children with heavy prenatal alcohol exposure. Poster presented at the annual meeting of the Research Society on Alcoholism, San Antonio, TX.

**Nguyen, T. T.**, Simmons, R. W., Levy, S. S., Thomas, J. D., & Riley, E. P. (2010, March). Regulation of sustained isometric force in children with heavy prenatal alcohol exposure. Poster presented at the annual SDSU Student Research Symposium, San Diego, CA.

**Nguyen, T. T.**, Madsen, S. K., Foland-Ross, L. C., Thompson, P. M., Nicoletti, M., Brambilla, P., Soares, J. C., Bearden, C. E. (2009, May). Corpus callosum morphology in pediatric bipolar disorder. Poster presented at the annual conference for the Society of Biological Psychiatry, Vancouver, BC.

**Nguyen, T. T.**, Miller, M., Tagabuel, C., Fischetti, C., Childers, S., & Kotoyan, R. (2008, April). Prenatal valproic acid animal model of autism: Beneficial effects of increased dietary choline during pregnancy. Poster presented at the annual USC Undergraduate Research Symposium, Los Angeles, CA. **Awarded the Most Innovative Poster Award by the USC Stevens Institute for Innovation*

Lyon, M., Kroh, J., & **Nguyen, T. T.** (2007, May). Prenatal stress animal schizophrenia model increases NOS-containing neurons in pedunculopontine/laterodorsal tegmental complex as in human schizophrenia. Poster presented at the annual meeting for the Society of Biological Psychiatry, San Diego, CA.

**Nguyen, T. T.**, & Kroh, J. (2007, April). Animal model of schizophrenia demonstrates increases in number of nitric oxide synthase-containing neurons in brainstem nuclei similar to those found in human schizophrenia. Poster presented at the annual USC Undergraduate Research Symposium, Los Angeles, CA.

Wofford, A., **Nguyen, T. T.**, Butler, K. (2006, April). Ability of prenatally administered choline in overcoming depressed response to a noxious stimulus in a prenatal stress animal model of schizophrenia. Poster presented at the annual USC Undergraduate Research Symposium, Los Angeles, CA.

ABSTRACT OF THE DISSERTATION

Choline Intervention in Children with Fetal Alcohol Spectrum Disorders

by

Tanya T. Nguyen

Doctor of Philosophy in Clinical Psychology

University of California, San Diego, 2015
San Diego State University, 2015

Professor Jennifer D. Thomas, Chair

Prenatal alcohol exposure results in a broad range of neuropsychological and behavioral impairments. Considering the long-lasting problems associated with fetal alcohol spectrum disorders (FASD), the development of effective treatment programs is critical. Choline, an essential nutrient, is important for healthy fetal brain development, and preclinical studies have demonstrated that choline supplementation can attenuate the severity of alcohol-related cognitive impairments. Given this evidence, this project aimed

to translate preclinical findings to a clinical population to investigate if choline supplementation can ameliorate the severity of memory, executive function, and attention deficits in children with FASD. The current study was a randomized, double-blind, placebo-controlled clinical trial that explored the effectiveness of a choline intervention for children with FASD ages 5-10 years. Fifty-five children with confirmed histories of heavy prenatal alcohol exposure were randomly assigned to either the choline ($n$ = 29) or placebo ($n$ = 26) treatment arms. Participants in the choline group received 625 mg choline daily for 6 weeks, whereas those in the placebo group received an equivalent dose of an inactive placebo treatment. Primary outcomes were assessed at baseline and post-intervention, including performance on neuropsychological measures of memory, executive function, and attention/hyperactivity. Results indicated that participants in the choline group did not differentially improve in cognitive performance in any domain, relative to the placebo group. Dietary choline intake was a marginally significant predictor of memory performance at post-assessment, suggesting a trend towards better memory performance in participants with higher levels of dietary choline intake. Overall, the findings of the present study do not support that choline, adminstered at a dose of 625 mg/day for 6 weeks, is an effective intervention for children with FASD. However, this study is only one of the first to translate preclinical findings to a human clinical trial of choline supplementation in FASD. Additional studies are needed to further elucidate whether choline and other nutritional supplements may improve outcome and various parameters which may influence effective translation. Altogether, this study highlights the need for a continued understanding of the role of nutritional status and supplementation in FASD and its contributions to neurocognition.

# I. INTRODUCTION

Fetal alcohol spectrum disorders (FASD) represent a range of outcomes resulting from alcohol exposure during pregnancy that disrupts fetal development (Bertrand et al., 2004). Prenatal alcohol exposure can result in a wide range of physical, cognitive, neurobehavioral, and social impairments. The most well known outcome of prenatal alcohol exposure is fetal alcohol syndrome (FAS), which is among the most severe outcomes on the FASD spectrum and characterized by pre- and/or postnatal growth deficits, distinct craniofacial dysmorphology, and central nervous system (CNS) dysfunction (Jones, Smith, Ulleland, & Streissguth, 1973). Still, many individuals may experience consequences of developmental exposure to alcohol, including damage to the developing brain and neurobehavioral deficits, even if they lack the facial features required for a diagnosis of FAS (Mattson, Riley, Gramling, Delis, & Jones, 1998).

FASD is a global health concern (Warren et al., 2001), estimated to affect as high as 2-5% of young school children in the United States and Western Europe (May et al., 2011; May et al., 2009) and 7-9% in South Africa (May, et al., 2009; May et al., 2007). Prenatal exposure to alcohol is one of the leading known preventable cause of birth defects, intellectual disability, and neurodevelopmental disorders around the world (Abel & Sokol, 1987; Pulsifer, 1996). Considering these prevalence rates, it is not surprising that FASD is associated with significant personal, social, and economic ramifications (Abel & Sokol, 1987; Lupton, Burd, & Harwood, 2004; Olson et al., 2009). However, despite known adverse consequences, public health warnings, and other prevention efforts, women continue to consume alcohol during pregnancy (Tsai et al., 2010),

particularly in patterns known to be of high risk to the developing fetus. For example, in the U.S., 7.6% of women report drinking during pregnancy and 1.4% report binge patterns of drinking consistently found to be damaging to fetal development (Centers for Disease Control and Prevention, 2012). Furthermore, approximately 51.5% of women of childbearing age report alcohol use, and among those, 15% report binge drinking (Centers for Disease Control and Prevention, 2012). As over 50% of pregnancies in the U.S. are unplanned (Finer & Henshaw, 2006), these women are of particularly high risk for giving birth to a child with an FASD. These data suggest that although prevention strategies to reduce the number of alcohol-exposed pregnancies are important, additional efforts must be made to develop effective, evidence-based intervention programs to ameliorate adverse consequences in children already affected by prenatal alcohol exposure.

**Consequences of Developmental Alcohol Exposure**

The outcomes resulting from prenatal alcohol exposure vary widely and can range from isolated organ damage or subtle developmental disabilities to FAS and stillbirth. In addition to growth deficiencies and facial dysmorphology (Hoyme et al., 2005)—features more often associated with a full FAS diagnosis—prenatal alcohol exposure leads to brain dysfunction and a broad range of neuropsychological and neurobehavioral impairments. Neurostructural and neurofunctional abnormalities have been extensively documented in the literature, including overall reduction in brain size, alterations in gray and white matter densities and integrity, and volumetric reductions in the cerebral cortex, corpus callosum, cerebellum, basal ganglia, and hippocampus (Coles & Li, 2011; Lebel,

Roussotte, & Sowell, 2011; Wozniak & Muetzel, 2011). These studies reveal that alcohol's effects during development are not only pervasive but also specific for certain brain regions. Likewise, the cognitive and behavioral deficits seen in FASD are global in nature but may also be specific, depending on affected brain regions. Neuropsychological studies suggest that individuals prenatally exposed to alcohol present with a wide range of impairments in a variety of domains, including diminished general intelligence, poor learning and memory, impaired executive and visuospatial function, hyperactivity and attention deficits, and delayed motor and language development (Mattson, Crocker, & Nguyen, 2011). Of these deficits, executive dysfunction and impaired spatial processing appear to be particularly sensitive to prenatal alcohol exposure and may be core deficits in FASD (Mattson et al., 2010).

Individuals with FASD suffer from many physical, cognitive, emotional, and social problems, which affect daily functioning and result in adverse life outcomes. In addition to the neuropsychological deficits already described, children with FASD are likely to present with clinically significant behavioral characteristics. Alcohol-exposed children have marked disturbances in behavioral and emotional functioning, including externalizing and internalizing behavioral problems, increased negative affect, and conduct problems (Coles, Platzman, Brown, Smith, & Falek, 1997; Coles, Platzman, & Lynch, 1999; D'Onofrio et al., 2007; Fryer, McGee, Matt, Riley, & Mattson, 2007; Mattson & Riley, 2000; Nash et al., 2006; O'Connor, 2001; O'Connor & Paley, 2006; O'Connor et al., 2002; Sood et al., 2001; Staroselsky et al., 2009; Steinhausen, Willms, Metzke, & Spohr, 2003). As a result of these behavioral alterations, these children are at higher risk for psychiatric diagnoses, including increased rates of mood disturbance as

well as oppositional defiant disorder, conduct disorder, and attention-deficit/hyperactivity disorder (ADHD) (Burd, Klug, Martsolf, & Kerbeshian, 2003; D'Onofrio, et al., 2007; Disney, Iacono, McGue, Tully, & Legrand, 2008; Fryer, et al., 2007; O'Connor & Paley, 2009; Steinhausen & Spohr, 1998; Steinhausen, Willms, & Spohr, 1993). These behavioral difficulties and psychopathologies often persist into adulthood (Barr et al., 2006; Famy, Streissguth, & Unis, 1998; Spohr, Willms, & Steinhausen, 2007) and result in secondary disabilities, including problems in school and work environments, inappropriate sexual behaviors, substance abuse problems, trouble with the law, difficulties with independent living, among other challenges (Alati et al., 2006; Alati et al., 2008; Baer, Barr, Bookstein, Sampson, & Streissguth, 1998; Baer, Sampson, Barr, Connor, & Streissguth, 2003; Fast, Conry, & Loock, 1999; Streissguth, Barr, Kogan, & Bookstein, 1996; Streissguth et al., 2004). Given the need for lifelong medical care services and loss of productivity, the lifetime cost a single individual with FASD was approximated to be between $2 million, including medical care services and lost productivity (Lupton, et al., 2004), placing substantial burdens on families and health care systems.

**Treatments and Interventions for FASD**

Considering the long-lasting medical, psychological, and social problems associated with prenatal alcohol exposure, developing effective treatment programs to improve cognitive and behavioral outcomes in individuals with FASD is critical. Since FAS was identified 40 years ago, surprisingly little is known about effective treatments (Premji, Benzies, Serrett, & Hayden, 2007). Only recently have a number of both animal

and human studies shown promising evidence that alcohol-related impairments may be responsive to intervention. The considerable variability of outcomes resulting from fetal alcohol exposure has made development of treatments particularly challenging, as a diagnosis of an FASD does not lend itself to a single treatment that can target the entire range of problems (Hannigan & Berman, 2000). Thus, the majority of interventions that have been investigated among individuals with FASD are problem-specific and symptom-focused. These programs have primarily emphasized educational, social skills, and cognitive training (Paley & O'Connor, 2009) and have shown some promise in reducing alcohol-related deficits in cognitive, social, and behavioral skills (e.g., Adnams et al., 2007; Bertrand, 2009; Coles, Kable, & Taddeo, 2009; Grant et al., 2004; Kable, Coles, & Taddeo, 2007; Loomes, Rasmussen, Pei, Manji, & Andrew, 2008; O'Connor et al., 2006). In addition to intervening with alcohol-exposed individuals, treatment practices have focused on providing education and support to families and caregivers to help improve parenting skills, increase parent self-efficacy, and reduce stress and strain on the parent-child relationship—all of which may promote positive developmental trajectories (Paley & O'Connor, 2009). Although the data seem promising, large-scale implementation of these programs has been limited, given challenges related to cost and resources needed for translation to community settings.

In addition to clinical studies, animal research has provided valuable insights into potential directions for interventions. Animal model systems suggest a variety of potential therapeutics and strategies for intervention that can attenuate a broader range of adverse outcomes due to prenatal alcohol exposure. A number of studies have shown positive effects of neonatal handling, postnatal environment enrichment, and

rehabilitative training in subjects with prenatal alcohol exposure (Hannigan, O'Leary-Moore, & Berman, 2007). Animal studies have also highlighted substances, such as neurotrophic factors, antioxidants, and nutrients that may protect against or attenuate alcohol's teratogenic effects.

## Nutrition and FASD

Understanding the risk and protective factors that may influence the development of children with FASD is critical for the development of effective prevention and intervention strategies. Nutrition is an important modifier of FASD risk. Nutrition interacts with alcohol in various ways that may potentially exacerbate or protect against alcohol's teratogenicity.

**Nutritional Deficiency**. Maternal alcohol use during pregnancy threatens the availability of important micronutrients critical for normal development of the fetus. Deficiencies may arise as a result of poor maternal dietary habits—as alcohol often alters caloric intake—or through alcohol-induced interference in the absorption, metabolism, and utilization of select nutrients (Dreosti, 1993). Poor maternal nutrition is a significant problem for mothers who drink alcohol during pregnancy, particularly in high-risk populations. Nutrition studies have shown that women in the Western Cape Province of South Africa, regardless of drinking status, have major nutritional deficiencies; however, mothers of children with FASD were reported to have significantly lower intake of riboflavin, calcium, omega-3 fatty acids, docosapentaenoic acid, and choline (May et al., 2004; May et al., 2014). Furthermore, an investigation of the nutritional status of pregnant women in Russia and the Ukraine revealed that alcohol-consuming mothers

have significantly lower levels of plasma zinc and copper when compared to nondrinking mothers attending the same prenatal clinics (Keen et al., 2010). Such nutritional deficiencies pose a severe threat to healthy fetal development. Evidence from animal models clearly demonstrate that undernutrition increases alcohol-related fetal toxicity (Shankar et al., 2006; Wainwright & Fritz, 1985; Weinberg, D'Alquen, & Bezio, 1990; Wiener, Shoemaker, Koda, & Bloom, 1981) and influences alterations in fetal gene expression (Shankar, Ronis, & Badger, 2007).

Although little is known about how prenatal alcohol exposure or maternal nutrition may affect the nutritional status of offspring, two recent investigations revealed that children with FASD display higher rates of disordered feeding behaviors and obesity (among girls only; average age of 8 years) and exhibit nutrient inadequacies (Fuglestad et al., 2013; Werts, Van Calcar, Wargowski, & Smith, 2014). In particular, Fuglestad and colleagues reported that children with FASD, ages 2.5-4.9 years, had lower dietary intakes of saturated fat, vitamin D, and calcium compared to their typically developing peers in the U.S., and that the majority of children did not meet the Dietary Reference Intakes (established by the Institute of Medicine) for fiber, omega 3 fatty acids, vitamin D, vitamin E, vitamin K, choline, and calcium. Therefore, these findings suggest that children with FASD may benefit from nutritional intervention.

**Nutritional Supplementation**. While nutritional deficiencies may convey increased risk for FASD, nutritional interventions may serve as a potential solution to reduce the adverse effects due to prenatal alcohol exposure. Preclinical studies have shown that dietary supplementation with nutrients such as zinc, folate, and antioxidants can provide a degree of protection against alcohol-induced teratogenesis (Antonio &

Druse, 2008; Cohen-Kerem & Koren, 2003; Serrano, Han, Brinez, & Linask, 2010; Summers, Henry, Rofe, & Coyle, 2008; Summers, Rofe, & Coyle, 2006, 2009; Wang et al., 2009). Nutritional supplements may compensate for changes in the bioavailability of nutrients due to alcohol metabolism (Lieber, 2000), as well as alcohol-related reductions in nutritional intake and absorption (Dreosti, 1993). However, nutritional supplements may also alter development, even if they do not target the same sites as alcohol-related actions. That is, nutrient supplementation may not necessarily directly limit alcohol's action but may stimulate growth and development through other pathways that may compensate for alcohol-related brain dysfunction. For example, zinc supplementation can mitigate spatial and object recognition memory impairments by preventing reductions in plasma zinc concentrations associated with gestational alcohol exposure, thereby increasing fetal access to zinc; however, zinc may also promote cell survival through anti-apoptotic mechanisms (Summers, et al., 2008), even with exposure to teratogens that do not affect zinc level (Fernandez, Gustafson, Andersson, Hellman, & Dencker, 2003). Thus, nutritional interventions may protect against alcohol-related impairments regardless of whether they are compensating for an alcohol-related nutritional deficiency (Idrus & Thomas, 2011).

Using a rodent model of FASD, our lab was the first group to examine the effects of a nutritional intervention of choline in subjects exposed to alcohol during development, revealing that choline can attenuate fetal alcohol effects (Thomas, La Fiette, Quinn, & Riley, 2000). Choline supplementation can reduce the severity of alcohol's adverse effects on behavioral development whether administered at the same time as prenatal alcohol exposure (Thomas, Abou, & Dominguez, 2009; Thomas, Idrus, Monk, &

Dominguez, 2010) or—perhaps more importantly—postnatally after alcohol exposure has occurred (Thomas, Biane, O'Bryan, O'Neill, & Dominguez, 2007; Thomas, et al., 2000). Choline is one of the few nutrients that has been shown to be effective even when administered long after alcohol exposure, during periods of development that would be equivalent to postnatal development in humans.

**Choline and Brain Development**

Choline is an essential nutrient necessary for the growth of mammalian cells and critical for both prenatal and postnatal brain development (Blusztajn, 1998; Zeisel & Niculescu, 2006). Choline serves as a precursor to membrane constituents (e.g., phospholipids, phosphatidylcholine, and sphingomyelin), signaling factors (e.g., platelet-activating factor and sphingosylphosphorycholine), and intracellular messengers (e.g., diacylglycerol and ceramide) as well as the neurotransmitter acetylcholine (Alkondon, Pereira, Cortes, Maelicke, & Albuquerque, 1997; Zeisel & Blusztajn, 1994). Moreover, choline is a precursor to betaine, which functions as a source of labile methyl groups that influence the methionine-homocysteine cycle. Thus, choline is required for many cellular processes affecting the integrity of cellular membranes, intracellular signaling, cholinergic neurotransmission, and gene expression (Davison, Mellott, Kovacheva, & Blusztajn, 2009; Mehedint, Niculescu, Craciunescu, & Zeisel, 2010; Niculescu, Craciunescu, & Zeisel, 2006; Zeisel, 2006, 2011b; Zeisel & Blusztajn, 1994).

Through deficiency and supplementation studies, animal research has highlighted the importance of choline during various developmental periods. In typically developing rodents, prenatal choline deficiency disrupts brain and cognitive development, producing

neural tube defects, decreasing progenitor proliferation, and increasing apoptotic cell death (Albright, Tsai, Friedrich, Mar, & Zeisel, 1999; Fisher, Zeisel, Mar, & Sadler, 2001, 2002; McKeon-O'Malley, Siwek, Lamoureux, Williams, & Kowall, 2003; Meck & Williams, 1997b). Furthermore, subjects that were prenatally deficient in choline showed diminished visuospatial memory as adults (Meck & Williams, 1999), suggesting that choline can have persistent effects on cognitive abilities into adulthood and that choline availability during early development is critical for functioning in later life. On the other hand, increased choline availability during early development leads to improved birth outcomes. Animal studies demonstrate that pre- and perinatal choline supplementation stimulates cell division and leads to long-lasting enhancements in the developing CNS (Albright, Friedrich, Brown, Mar, & Zeisel, 1999). Specifically, choline supplementation has been shown to induce structural and functional changes in the hippocampus and cortex that continue into adulthood, long after the period of supplementation has ended. Some of these changes include increased cell division, cell size, and dendritic arborization (Albright, Friedrich, et al., 1999; Albright, Mar, Craciunescu, Song, & Zeisel, 2005; Albright, Tsai, Mar, & Zeisel, 1998; Craciunescu, Albright, Mar, Song, & Zeisel, 2003; Li et al., 2004; Loy, Heyer, Williams, & Meck, 1991; Meck & Williams, 2003; Williams, Meck, Heyer, & Loy, 1998; Zeisel & Niculescu, 2006), increased growth and neurotrophic factors (Mellott et al., 2007; Napoli, Blusztajn, & Mellott, 2008; Sandstrom, Loy, & Williams, 2002), more efficient cholinergic neurotransmission (Blusztajn, Cermak, Holler, & Jackson, 1998; Cermak et al., 1999; Cermak, Holler, Jackson, & Blusztajn, 1998; Coutcher, Cawley, & Wecker, 1992; Li, et al., 2004; Meck, Smith, & Williams, 1989; Montoya & Swartzwelder, 2000; Montoya et al., 2000) and long-term

potentiation (Jones, Meck, Williams, Wilson, & Swartzwelder, 1999; Pyapali, Turner, Williams, Meck, & Swartzwelder, 1998), and increased adult neurogenesis (Glenn et al., 2007). Epidemiological data from a large population-based case-control study are consistent with these preclinical findings, revealing that maternal diets containing higher levels of periconceptional dietary choline and betaine were associated with decreased risk of neural tube defects in infants (Shaw, Carmichael, Yang, Selvin, & Schaffer, 2004).

Choline modifications in brain development directly translate to behavioral effects both prenatally and postnatally. Choline deficiency leads to long-term impairments in cognition, whereas choline supplementation leads to long-lasting improvements in spatial, temporal, and reversal learning and memory as well as sustained attention (see McCann, Hudes, & Ames, 2006 for a review). Such effects of improved cognition are evident even in aged rats, long after choline supplementation has ended (Cheng, Scott, Penney, Williams, & Meck, 2008; Meck & Williams, 1997a, 2003; Meck, Williams, Cermak, & Blusztajn, 2007), illustrating that choline can protect against the effects of normal cognitive aging. In addition, animal models have demonstrated that choline supplementation can protect against other disorders and insults, including Down syndrome (Moon et al., 2010), Rett syndrome (Nag & Berger-Sweeney, 2007; Nag, Mellott, & Berger-Sweeney, 2008; Ward, Kolodny, Nag, & Berger-Sweeney, 2009), traumatic brain injury (Guseva, Hopkins, Scheff, & Pauly, 2008), Alzheimer's disease-related pathology and dementia (Fu et al., 2004), NMDA-induced toxicity (Mulholland, Self, Harris, Littleton, & Prendergast, 2004), MK-801-induced toxicity (Guo-Ross et al., 2002; Guo-Ross, Jones, Shetty, Wilson, & Swartzwelder, 2003), and kainic acid-induced status epilepticus (Holmes et al., 2002; Wong-Goodrich et al., 2011). Given the important

role of choline, the Food and Nutrition Board of the Institute of Medicine (IOM; Institute of Medicine, 2006) recommends adequate choline intake levels for pregnant and lactating women, and choline is now added to prenatal vitamins as well as foods and multivitamins targeted for children (Zeisel, 2009; Zeisel & da Costa, 2009).

## Choline Supplementation and Developmental Alcohol Exposure

**Preclinical Studies**. Given the data on the effects of choline supplementation on typical development, our lab has examined the possibility that choline may serve as an effective treatment for FASD. Evidence from these preclinical studies suggest that choline supplementation can improve cognitive outcomes among animals exposed to alcohol during development, even when administered postnatally after alcohol exposure has occurred (Ryan, Williams, & Thomas, 2008; Thomas, et al., 2009; Thomas, et al., 2007; Thomas, Garrison, & O'Neill, 2004; Thomas, et al., 2010; Thomas, et al., 2000; Thomas & Tran, 2012). In particular, choline mitigates behavioral deficits on tasks that depend on the functional integrity of the hippocampus and prefrontal cortex, including impairments in spatial learning and memory (Thomas, et al., 2004), hyperactivity (Thomas, et al., 2004), trace classical conditioning (Thomas & Tran, 2012; Wagner & Hunt, 2006), and working memory (Thomas, et al., 2000). More specifically, when administered during postnatal day (PD) 11-20 or PD 21-30 in the developing rat (i.e., developmental windows that may correspond to early and middle childhood, respectively), choline significantly reduces the severity of spatial learning and object recognition deficits (Ryan, et al., 2008). These data suggest that choline could effectively reduce the severity of alcohol's teratogenic effects even when administered later in

development. In fact, even when choline is administered from PD 40-60 (i.e., a period equivalent to adolescence/young adulthood in humans), it significantly attenuates alcohol-related working memory deficits (Schneider, Dominguez, & Thomas, 2008). Interestingly, during this later developmental period (PD 40-60), choline did not mitigate alcohol-related overactivity in the open field or deficits in simple spatial learning, suggesting that choline may target the prefrontal cortex more than the hippocampus as development progresses and the brain matures.

Research supports that the hippocampus and prefrontal cortex are adversely affected in children following prenatal alcohol exposure (Astley et al., 2009; Nardelli, Lebel, Rasmussen, Andrew, & Beaulieu, 2011) and that the neuropsychological domains of learning and memory, visuospatial ability, attention, and executive function are significant areas of deficit in children with FASD (Mattson, et al., 2011). Early clinical case studies in neurodevelopmentally delayed children demonstrate improvement in these domains with choline supplementation (Woodbury & Woodbury, 1993), and these areas are expected to be most beneficially affected by choline supplementation in children with FASD.

**Mechanisms of Choline's Effects**. Despite preclinical evidence that choline supplementation can attenuate alcohol's adverse effects on development, the mechanisms by which choline improves cognitive function have yet to be elucidated. As previously discussed, choline serves many biological functions and can affect development through several different processes, including cholinergic transmission, cellular membranes, signaling pathways, and gene expression (Zeisel, 2006; Zeisel & Blusztajn, 1994). Some evidence indicates that choline supplementation during early development may lead to

metabolic imprinting of cholinergic networks in the basal forebrain, hippocampus, and frontal cortex (Meck & Williams, 2003), important brain regions involved in memory processing and other cognitive functions. Alcohol exposure leads to abnormal hippocampal cholinergic development in alcohol-exposed animals, and research has demonstrated that choline supplementation (PD 4-30) attenuates some of these effects (Monk, Leslie, & Thomas, 2012). In addition to the cholinergic pathway, choline supplementation (PD 2-21) has been found to reduce alcohol-related hypermethylation in the hippocampus and prefrontal cortex (Otero, Thomas, Saski, Xia, & Kelly, 2012), suggesting the possibility that choline's protective effects during development may be mediated by epigenetic modification of fetal gene expression. These animal models indicate that choline likely has multiple sites of action, and the mechanisms by which it may attenuate alcohol-related insults likely depend on the timing and duration of treatment.

**Clinical Studies**. Based on preclinical data, three randomized clinical trials are currently investigating the effects of choline supplementation in populations with FASD or at risk for FASD. The first study is exploring micronutrient supplementation with or without choline among alcohol-consuming pregnant women in the Ukraine (Keen, et al., 2010). Preliminary findings from this study have revealed that micronutrient supplementation improves mental development in infants prenatally exposed to alcohol (as measured by the Bayley Scale of Infant Development), although choline did not improve outcomes beyond micronutrients alone (Coles et al., 2011). Additional data suggest that choline may improve neurophysiological encoding and memory (i.e., habituation) in alcohol-exposed infants independent of micronutrient supplementation

alone at 6-months (Kable et al., 2011) and 12-18 months (Kable et al., 2012), although these data are from intermediary analyses, and final results have yet to be published. Another similar study is examining prenatal choline supplementation among alcohol-consuming pregnant women in the Western Cape Province of South Africa. The third study is investigating the effect of a 9-month choline supplementation in young children with FASD aged 2-4 years (Wozniak et al., 2013; Wozniak et al., 2011). Feasibility and tolerability data from Phase 1 of this study demonstrated compliance rates between 82-87% and minimal adverse events that were equivalent in the choline and placebo treatment arms. Examination of efficacy data is currently underway. While these studies will provide data on the effects of choline supplementation in early prenatal development through early childhood, additional research is needed as children continue to age and a wider range of behaviors can be examined.

In addition to studies investigating choline supplementation in prenatal alcohol exposure, a number of clinical trials are currently underway investigating the effects of choline supplementation in both normal and clinical populations. Several recruiting and ongoing studies are evaluating the effectiveness of choline supplementation during pregnancy on infant cognitive development (The Gerber Foundation & Egg Nutrition Center; University of Colorado Denver), and other clinical trials are investigating the ability of dietary choline to improve nutrient absorption in children with cystic fibrosis and pancreatic insufficiency (Avanti Polar Lipids Inc. & National Institute of Diabetes and Digestive and Kidney Diseases; University of British Columbia & Cystic Fibrosis Foundation), liver function in hepatic steatosis patients (FDA Office of Orphan Products

Development & University of Texas), and core symptoms in children with autism spectrum disorders (Sheba Medical Center & the Israeli Society of Clinical Pediatrics).

**Purpose of the Current Study**

Although much research has demonstrated the clinical potential of choline and other nutritional interventions in alcohol-exposed subjects, the majority of this work is still only in the preclinical phase. Various nutritional interventions have been studied in children with autism, ADHD, and Down's syndrome, including antioxidants, vitamins (e.g., vitamins A, $B_6$, and C, and multivitamin combinations), minerals (e.g., magnesium, zinc), carnosine, folate, and carnitine (Adams & Holloway, 2004; Akhondzadeh, Mohammadi, & Khademi, 2004; Amminger et al., 2007; Bilici et al., 2004; Chez et al., 2002; Dolske, Spollen, McKay, Lancashire, & Tolbert, 1993; Ellis et al., 2008; Harding, Judah, & Gant, 2003; Joshi et al., 2006; Mousain-Bosc et al., 2006; Mousain-Bosc, Roche, Rapin, & Bali, 2004; Richardson & Montgomery, 2005; Richardson & Puri, 2002; Rimland, 1988, 1998a, 1998b; Sinn & Bryan, 2007; Stevens et al., 2003; Van Oudheusden & Scholte, 2002), yet the translation of nutritional interventions to children with FASD has only recently been investigated. In addition to the aforementioned ongoing clinical studies exploring choline supplementation individuals with or at risk for FASD, the only other study that has attempted nutritional supplementation in a clinical population exposed to prenatal alcohol exposure investigated mega-doses of vitamin C and E in pregnant women who drank alcohol and was prematurely terminated for safety concerns (Goh, Ungar, Rovet, & Koren, 2007).

The current study is a randomized, double-blind, placebo-controlled clinical trial that explored the effectiveness of a 6-week postnatal choline supplementation administered to children with FASD ages 5-10 years. This study sought to extend the other ongoing studies by examining whether choline is effective among school-aged children with FASD in order to understand the effects of choline across the early lifespan, from infancy through later childhood. Although early interventions are needed, FASD is not often identified until children enter school (Olson, Jirikowic, Kartin, & Astley, 2007); therefore, it is critical to examine treatment options for children at older ages. Examination of a nutritional intervention during later development has important implications for children with prenatal alcohol exposure who are identified later in life.

The primary aims of this study were to investigate if choline supplementation in children with FASD could ameliorate the severity of learning and memory, executive function, and attention deficits among children with FASD. These are areas of known deficit among this population and are hypothesized to improve following choline intervention, given the well-documented associations between choline and brain development in hippocampus and prefrontal cortex, as well as preclinical evidence demonstrating improvements in neurobehavioral domains associated with these brain regions. Specifically, in the domain of memory, it was hypothesized that children would demonstrate improved nonverbal object recognition and visuospatial memory (Aim 1). With regards to executive function, it was hypothesized that choline intervention would ameliorate impairments in the domains of inhibitory control, cognitive flexibility, working memory, and planning (Aim 2). Finally, in the domain of attention, it was expected that choline supplementation would improve sustained attention as well as

reduce hyperactivity and impulsivity in children with FASD (Aim 3). In contrast, a task of psychomotor abilities was included as a negative control, as fine motor coordination was expected to be sensitive to prenatal alcohol exposure but not to choline supplementation (Aim 4). Although animal studies have demonstrated that choline supplementation can attenuate deficits in behaviors mediated by forebrain cholinergic systems, postnatal choline supplementation does not significantly attenuate alcohol-related deficits in motor coordination or delay eyeblink conditioning, which are dependent on the cerebellum (Thomas, et al., 2004; Thomas & Tran, 2012). This aim would demonstrate whether choline's effects during this period of development are specific to the hippocampus and/or cortex and not the cerebellum. Finally, secondary analyses were conducted to explore the effect of moderators on treatment outcomes. We hypothesized that higher levels of treatment compliance and lower levels of dietary choline would be associated with improved cognitive performance in the choline group.

Chapter I, in part, is currently being prepared for submission for publication of the material. Nguyen, T. T.; Risbud, R. D.; Chambers, C.; & Thomas, J. D. The dissertation author was the primary investigator and author of this material.

# II. METHODS

## Participants

Participants included 55 children with confirmed histories of heavy prenatal alcohol exposure between the ages of 5:00 and 10:11 years. Individuals were recruited through and assessed at two primary sites: (1) Center for Behavioral Teratology (CBT) at San Diego State University and (2) Double ARC, a nonprofit organization providing services to families of children with FASD in Toledo, Ohio. Children were also recruited through the Genetics and Dysmorphology Clinic at Rady Children's Hospital–San Diego, postings on websites and listservs for families of children with FASD, and ClinicalTrials.gov.

Upon initial telephone contact, caregivers of prospective participants were provided with information about the intervention and the requirements of their participation. At that time, they also completed a brief telephone-screening interview to provisionally determine their appropriateness for study participation. Caregivers were informed that participation would include random assignment into an experimental protocol designed to test a nutritional intervention that may reduce cognitive symptoms related to prenatal alcohol exposure. Participants who qualified based on this preliminary screening were invited to complete further assessment to confirm eligibility. Final eligibility for the study was determined by T.N.

Eligible participants were required to meet criteria for heavy prenatal alcohol exposure and be primary English speakers between the ages of 5-10 years at the time of study enrollment. Heavy exposure was defined as at least 4 drinks per occasion at least

once per week or at least 14 drinks per week during pregnancy. History of prenatal alcohol exposure was determined retrospectively through a review of available medical, social service, or adoption records as well as maternal and/or family/friend report, when available. In many cases when detailed information about timing, duration, and quantity of alcohol consumption was unavailable, mothers were reported to be "alcoholic," alcohol abusing, or alcohol dependent during pregnancy. Thirty-one participants received a dysmorphology examination conducted by Dr. Kenneth Lyons Jones to determine FAS diagnosis based on physical, craniofacial, and growth anomalies; FAS was defined by the presence of two or more key facial features (short palpebral fissures, smooth philtrum, thin vermillion), growth deficiency (≤10th percentile for height or weight), and head circumference ≤10th percentile(for more details, see Jones et al., 2006). Additionally, 7 participants had received a formal diagnosis of an FASD (i.e., including a dysmorphological and physical exam) from other sources (e.g., by dysmorphologists other than Dr. Jones; use of different diagnostic systems, such as the 4-Digit-Code). Exclusion criteria included history of significant head injury with loss of consciousness greater than 30 minutes, significant physical (e.g., uncorrected visual impairment, hemiparesis), neurologic (e.g., seizure disorder), or psychiatric (e.g., active psychosis) disability that precluded involvement in the study, evidence of any other known causes of mental deficiency (e.g., congenital hypothyroidism, neurofibromatosis, chromosomal abnormalities), or prescription of medications that were suggestive of or might increase risk for atherosclerosis, such as beta-blockers, estrogen, progesterone, or testosterone supplements, protease inhibitors, and long term systemic prednisone and cyclosporine.

Medication changes during the treatment period did not preclude study participation but were monitored.

In total, caregivers of 91 children inquired or were contacted about the program, and 81 were screened for participation. Of those, 13 declined to further participate and 10 were deemed ineligible. Of those excluded because they did not meet eligibility criteria, 4 had histories of seizure disorder, 1 had significant neurological concerns, 1 had a chromosomal disorder with associated risk of neurocognitive impairment, 1 had cerebral palsy, and 3 did not meet criteria for heavy prenatal alcohol exposure or had ambiguous histories that could not be used to determine degree of alcohol exposure. Fifty-eight participants were eligible and consented to participate, and these children were randomized to the choline or placebo conditions. Of those who were randomized, 3 were removed because they did not attend the baseline assessment session and did not initiate treatment. Of the 55 participants who initiated treatment, 3 were lost to follow up; 1 was unable to be contacted to schedule the post-assessment session and 2 were unavailable for the post-assessment session. **Figure 1** presents the flow diagram of participants through the phases of the study (i.e., enrollment, intervention allocation, follow-up, and data analysis) according to CONSORT guidelines for reporting clinical trials (Moher et al., 2010).

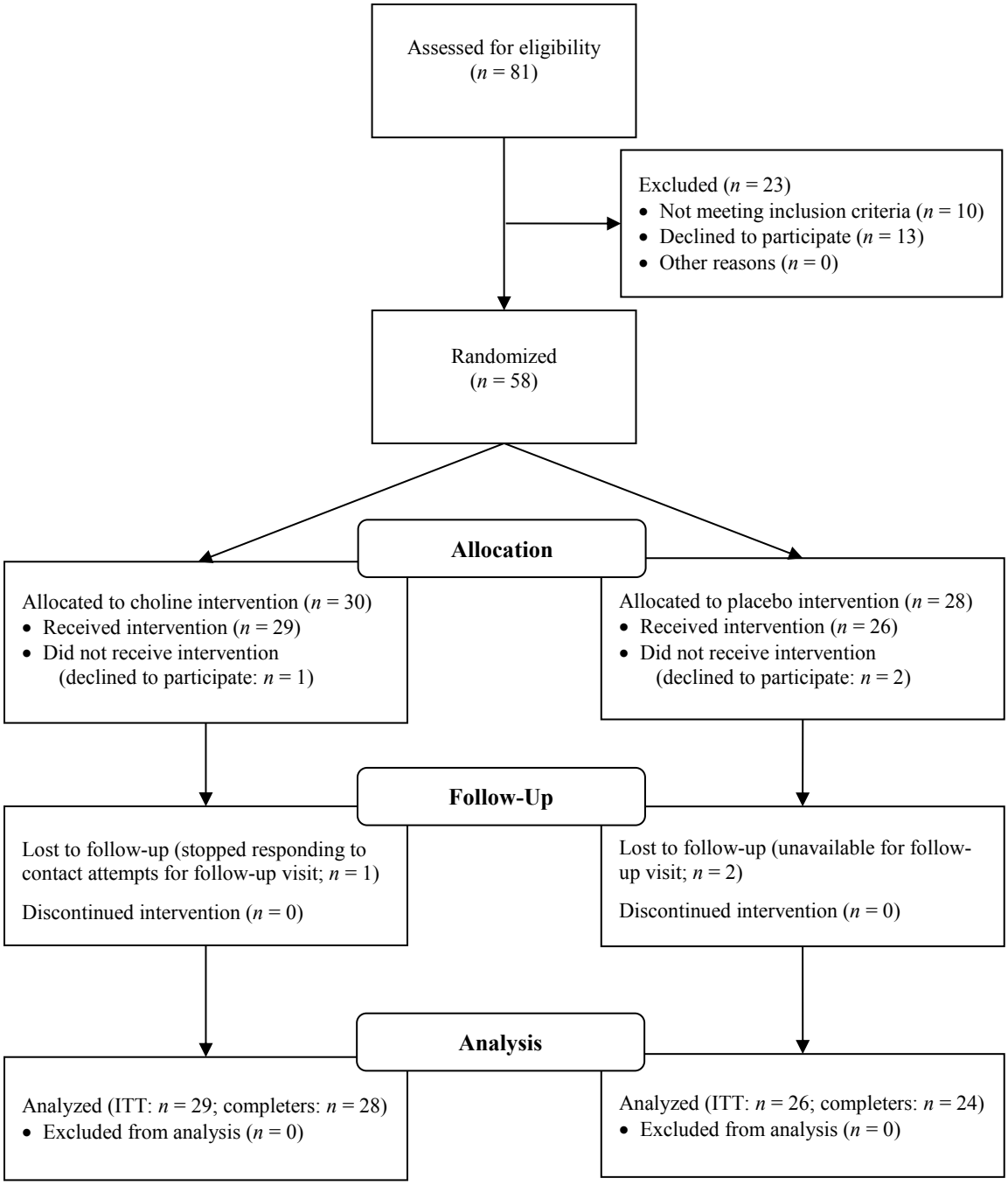


**Figure 1**. Flow diagram of the progression of participants through the study

## Procedures

The project and all procedures were approved by the Institutional Review Boards at San Diego State University and the University of California, San Diego.

**Allocated Intervention**. Following enrollment, participants were randomly assigned to the choline intervention or placebo intervention in a 1:1 allocation ratio based on a computer-generated random number generator. Randomization was stratified by gender (male and female) and age (5-6 years, 7-8 years, and 9-10 years) and followed a blocked design with variable block sizes (each block comprising of 2, 4, or 6 randomly ordered treatment assignments) to reduce selection bias. Randomization was handled independently so that participants and research personnel remained blind to group assignment.

Children in the choline group received 625 mg of choline daily for 6 weeks, orally administered in the form of a glycerophosphocholine (GPC) liquid concentrate (5.25 ml/day)(purchased from Nutrasal). GPC is a natural choline compound, prepared from the hydrolysis of phosphatidylcholine, and serves as a precursor to free choline. As the physiological form of choline in the body, GPC can rapidly cross the blood-brain barrier due to its water solubility. GPC is an important component in human breast milk (Holmes-McNary, Cheng, Mar, Fussell, & Zeisel, 1996) and is found in many common food items, such as dairy products, olive oil, oat bran, and liver (Zeisel, Mar, Howe, & Holden, 2003). The prescribed dose was 1.7-2.5 times the Adequate Intake recommended by the Food and Nutrition Board of the IOM (Institute of Medicine, 2006), depending on the child's age (see **Table 1**). Children in the placebo group received an equivalent dosage (5.25 ml/day) of an oral placebo treatment, consisting of a liquid mixture of

glycerin, water, and xylitol sweetener (also purchased from Nutrasal), daily for 6 weeks. The placebo treatment matched the GPC product in taste and consistency.

**Table 1**. Summary of choline Dietary Reference Intakes outlined by the Institute of Medicine

| Age Range | Adequate Intake (AI) Level (mg/day) | Prescribed Dose (mg/day) | Upper Intake Level (mg/day) |
|---|---|---|---|
| 4-8 years | 250 | 625 (2.5x AI) | 1000 |
| 9-13 years | 375 | 625 (1.7x AI) | 2000 |
| 14-18 years | 550 males; 400 females | N/A | 3000 |
| 19+ years | 550 males; 425 females | N/A | 3500 |

**Baseline Assessment Session**. Prior to the initial assessment, caregivers were instructed to refrain from administering any cognitive enhancement (e.g., stimulant) medications to their children on days of neuropsychological testing. Despite instructions, 4 children (2 placebo, 2 choline) were on cognitive medications on the morning of testing; to maintain consistency, caregivers of these children were asked to administer cognitive medications for the follow-up post-assessment visit. Upon arrival, caregivers and participants were informed about the study procedures and provided with written informed consent and assent, respectively. Participants were administered a standardized neuropsychological test battery lasting 80-95 minutes, consisting of tasks measuring cognitive abilities in the domains of memory, executive function, attention, and fine motor functioning, and their caregivers completed a 24-hour dietary recall interview. Following assessment, participants were provided with their allocated intervention, which was supplied in five 59 ml bottles containing a sweet-tasting liquid. Bottles were labeled with participants' identification numbers, in order to monitor consumption, and contained

no identifying information about the treatment (i.e., GPC or placebo), other than a code that allowed research personnel to dispense bottles blindly; in other words, all research personnel, caregivers, and participants were blind to group assignment.

Caregivers were instructed to administer 5.25 ml of the treatment daily for 6 weeks, either alone or by mixing with a beverage. They were specifically instructed to ensure that the treatment was completely consumed.

**Post-intervention Assessment Session**. Six weeks after the initial baseline session, participants returned to complete the post-assessment. Given research suggesting that plasma free choline levels peaks around three hours following oral administration of GPC (de Moliner, Abbiati, & Colombo, 1993), caregivers were instructed to administer the last treatment dose as close to three hours prior to the post-assessment appointment time as possible. At follow-up, 5 children (3 placebo, 2 choline) were on cognitive medications on the morning of testing. Participants completed the same neuropsychological test battery as was administered during the baseline session, and caregivers completed the 24-hour dietary recall interview.

**Monitoring Compliance**. Caregivers were provided with a treatment diary to record the date and time each dose was administered or the reason why it was missed. Caregivers were also instructed to save all treatment bottles (empty or filled) and bring them to the post-assessment session. The remaining liquid in bottles was measured.

**Adverse Events and Safety Management**. Families were contacted weekly by T.N. to assess for any problems with treatment administration and monitor any adverse events. Adverse events were assessed using a standardized protocol. Caregivers were asked general, open-ended questions about whether they had noticed any adverse events

or side effects since beginning treatment. Subsequently, they were prompted about the occurrence of specific symptoms (e.g., upset stomach, fishy body odor). Additionally, caregivers were asked if they had noticed any positive events since beginning treatment. All adverse and positive events were recorded.

Prior to study initiation, a Data Safety Monitoring Board (DSMB) was established to monitor adverse side effects. The DSMB consisted of an independent group of experts who advised the investigators in matters involving participant safety. Members of the DSMB included Dr. Loki Natarajan (chair), a biostatistician from UCSD who is involved in clinical trial design of Phase I trials and served as the DSMB chairperson of the prenatal micronutrient/choline supplementation study in the Ukraine (Keen, et al., 2010); Dr. Mari Golub, a developmental neurotoxicologist from the University of California, Davis who has served on a number of national advisory committees in the area of neurodevelopment, reproductive and developmental toxicity, and behavioral assessment, including the Ukraine prenatal supplementation study; and Dr. Judith Eckerle, a pediatrician specializing in FASD and adoption medicine from the University of Minnesota who is involved in the postnatal choline supplementation study (Wozniak, et al., 2013). The DSMB convened quarterly via teleconference to review and evaluate accumulated data on adverse events reported, any issues or concerns of participant safety, and study conduct, progress, and efficacy; they also provided recommendations concerning continuation, modification, or termination of the trial. Throughout the course of the study, the DSMB met 5 times, and on each occasion, unanimously supported continuation of the study. In addition to the DSMB, Dr. Howard Taras, a Professor of Pediatrics and Board-Certified Pediatrician at the University of California, San Diego

served as a safety consultant, advising investigators regarding individual participant concerns about adverse events.

## Measures

**Demographics**. Information about subject age, gender, race, ethnicity, handedness, and home placement was collected through caregiver report. Body weight was measured using a scale, and body weight percentile was calculated using growth charts, comparing participants' weight to that of other children in the same age group and gender. Socioeconomic status (SES) was assessed with the clinician-rated, Hollingshead 4-Factor Index of Social Position. The index assesses caregivers' gender, marital status, highest level of formal education, and current occupation. These factors are weighted, summed, and combined into a continuous measure of social index. Scores range from 8 to 66, higher scores are indicative of higher SES (Hollingshead, 1975).

**Compliance**. Information about treatment adherence was collected through treatment diaries completed by caregivers and by measuring remaining liquid volume in treatment bottles upon study completion. Caregivers reported each day whether the treatment was taken or missed. Full and empty treatment bottles were collected at study completion. Remaining liquid was measured using a graduated cylinder, and calculations were performed to determine the amount of treatment consumed and missed throughout the treatment period.

**Adverse Events**. Adverse events were assessed weekly throughout the treatment period through telephone visits. An adverse event was defined as any symptom, sign, illness, or experience that develops or worsens in severity during the course of the study;

intercurrent illnesses or injuries were also regarded as adverse events. A serious adverse event is any adverse event that is fatal, life-threatening, requires or prolongs hospital stay, results in persistent or significant disability or incapacity, a congenital anomaly or birth defect, or an important medical event (i.e., one that may not be immediately life threatening but may jeopardize the subject and/or may require intervention to prevent one of the other serious outcomes previously noted). Adverse events were classified into 11 categories: general health, skin, ear/nose/throat, cardiovascular, respiratory, gastrointestinal, genitourinary, musculoskeletal, neurologic, behavioral, and allergy. In addition to adverse events, positive events or changes that were reported by caregivers were recorded.

**Dietary Intake**. Nutrient intake consumed in subjects' diets was collected twice, at baseline and post-intervention, using the *Automated Self-administered 24-hour Dietary Recall* (ASA24; Subar et al., 2012), which provided information about dietary levels of choline as well as other vitamin and minerals. The ASA24 is a web-based application that guides respondents through the completion of a 24-hour dietary recall for the previous day. Caregivers were asked to report all foods, drinks, and dietary supplements consumed by their children in the previous day from a list of food and drink terms derived from the National Health and Nutrition Examination Survey (NHANES). The ASA24 asks detailed questions about food preparation, portion size, and additions so that food codes from the U.S. Department of Agriculture (USDA) Food and Nutrient Database for Dietary Surveys (FNDDS) can be assigned. Detailed information and analysis is provided about individual-level nutrients and food group estimates in an individual's diet based on the USDA Food and Nutrient Database for Dietary Surveys, the corresponding USDA

MyPyramid Equivalents Database, and the NHANES Dietary Supplement Database. Data on 10 macronutrients, 12 vitamins/micronutrients, and 8 minerals were obtained and included for analyses. Caregivers were instructed not to change their children's dietary supplements during the study. Concomitant supplement use was recorded at all study visits.

**Memory**. Nonverbal object and visuospatial memory was assessed using three subtests of the Cambridge Neuropsychological Test Automated Battery (CANTAB; Cambridge Cognition, 2006). The CANTAB is a computerized, touch-screen system. The CANTAB test battery is frequently utilized in child psychopharmacological studies because of its extensive validation, availability in parallel forms for repeat testing, and sensitivity to pharmacological manipulation (Curtis, Lindeke, Georgieff, & Nelson, 2002; Luciana & Nelson, 1998; Rhodes, Coghill, & Matthews, 2006). Subtests of the CANTAB have moderate to high test-retest reliability, with Pearson's *r* and Spearman's rho correlation coefficients ranging from .50 to .87 (DMS = .50, PRM = .72, PAL = .68 to .87, SWM = .63-.70, and SSP = .60).

***Delayed Matching to Sample (DMS)***. DMS is a forced-choice test of recognition memory of novel nonverbal stimuli. Participants were presented with a complex visual pattern followed by four similar patterns, from which the participant must select the pattern that exactly matched the sample previously displayed. In some trials the sample and the choice patterns are shown simultaneously, whereas in others a delay (0, 4, or 12 seconds) is introduced between covering the sample pattern and showing the choice patterns. Participants completed 20 counterbalanced trials. The dependent variable was

number of items correct for delay items. Higher scores are indicative of better memory performance.

***Pattern Recognition Memory (PRM)***. PRM is a test of visual pattern recognition memory in a two-choice forced discrimination paradigm. Participants were presented with a series of 12 visual patterns, one at a time. Then, in the recognition phase, pairs of patterns were presented, and participants were required to choose between a pattern previously seen and a novel pattern. A total of 24 items were completed. The dependent variable was number of items correct. Higher scores are indicative of better memory performance.

***Paired Associates Learning (PAL)***. PAL assesses the ability to recall nonverbal stimuli as well as spatial location simultaneously. In this task, boxes were displayed on the screen and opened in a randomized order, one or more of which contained a pattern. Participants were required to remember patterns associated with different locations on the screen. As each pattern was presented, participants selected the box where the pattern was originally located. Participants completed two items with a single pattern presented, two items with two patterns, two items with three patterns, one item with six patterns, and one item with eight patterns. If an item was not correctly completed within ten presentations, the test was discontinued. The dependent variable was total number of errors on the task, adjusted for stages not completed. Lower scores are indicative of better memory performance.

**Executive Function**. Four domains of executive function were assessed, including inhibitory control, cognitive flexibility, working memory, and planning.

***NEPSY-2 Inhibition***. Inhibitory control was assessed using the Inhibition subtest from the NEPSY-2 (Korkman, Kirk, & Kemp, 2007a). This task is a modified Stroop task, in which words and colors have been substituted for shapes and arrows, which is more appropriate for assessment of younger children with limited reading skills. Given the age and cognitive limits of the sample, only the Naming and Inhibition conditions were administered. Participants were presented with shapes (circles and squares) or arrows (pointing up and down). In the Naming condition, participants were asked to name the items as they were seen on the page (e.g., say "circle" for a circle, say "up" for an arrow pointing up). In the Inhibition condition, participants were asked to name the opposite of the items seen on the page (e.g., say "square" for a circle, say "down" for an arrow pointing up). Two trials (i.e., shapes and arrows) were completed for each condition. The dependent variable was number of errors on the Inhibition condition. Lower scores are indicative of better response inhibition. The Naming and Inhibition conditions of NEPSY-II Inhibition have high test-retest reliability coefficients ranging from .79 to .82 for children in the age group of 5-8 years (Korkman, Kirk, & Kemp, 2007b) and relatively minor practice effects (Brooks, Sherman, & Strauss, 2010).

***NEPSY-2 Design Fluency***. Cognitive fluency and flexibility were assessed using the Design Fluency subtest from the NEPSY-2. Participants drew as many unique designs as possible in a 60-second time limit, from both structured and unstructured dot arrays. The dependent variable was total number of correct designs across both structured and unstructured conditions. Higher scores are indicative of better cognitive flexibility.

***Spatial Working Memory (SWM)***. SWM is a test of the ability to retain visuospatial information in working memory. It is a self-ordered task, which also assesses

planning and problem solving. In this task, a number of boxes were presented on the screen, and participants were required to find a "token" in each of the boxes. Once a token was found in a box, it would not be presented there again. Using a process of elimination, participants collected "tokens" to fill up an empty column on the right hand side of the screen. An effective strategy for this task was to follow a predetermined sequence, beginning with a specific box and then, once a token has been found, returning to that box to start a new search sequence. Participants completed four items each with four boxes, six boxes, and eight boxes. Dependent variables were number of errors and strategy score. Lower number of errors is indicative of better working memory performance. Strategy score was obtained by counting the number of times a participant begins a new search with a different box. Higher scores represent poorer use of strategy and, consequently, inefficient planning.

***Spatial Span (SSP)***. SSP is a test of spatial attention and working memory capacity. It is a computerized version of the Corsi Blocks task and visuospatial analogue of the Digit Span test. Participants were shown a pattern of boxes on the screen. Some of the boxes changed color, one at a time, in a variable sequence. At the end of the presentation of each sequence, participants were required to select the boxes that changed color in the same order that they were displayed by the computer. The number of boxes in the sequence is increased from a level of 2 to 9, with three items at each level. The test discontinued if the participant failed on all three items at a particular level. The dependent variable was span length. Higher scores are indicative of better spatial attention and working memory.

**Attention**. Inattention, impulsivity, and hyperactivity—the core symptoms of ADHD—were assessed using the Quotient ADHD System (Teicher, Ito, Glod, & Barber, 1996).

***Quotient ADHD System***. The Quotient is a computerized system that measures motor hyperactivity during a continuous performance attention task (CPT). The task uses a Go/No-Go paradigm in which participants were presented with one of two geometric shapes in spatially random positions and asked to respond when the target shape appears and withhold response when the non-target shape appears. The task duration was 15 minutes. During the task, an infrared motion analysis system tracked and recorded the two-dimensional location of a reflective marker placed in the center of a headband worn on participants' heads. Dependent variables were traditional CPT measures of attention and movements variables from the motion tracking system. Measures assessed participants' (1) hyperactivity (e.g., movements, immobility duration, displacement, area), (2) inattention (e.g., accuracy, omission errors, response latency), and (3) impulsivity (e.g., commission errors).

**Motor**.

***Grooved Pegboard***. Psychomotor function was assessed using the Grooved Pegboard, a task of manual dexterity, hand-eye coordination, and fine motor speed. The dependent variable was completion time for dominant and non-dominant hands. Lower (more negative) scores indicate better motor performance.

A description of primary cognitive outcome measures and variables included in the analyses are presented in **Table 2**. All scores used in analyses were raw scores with the exception of Grooved Pegboard, which were age-corrected z-scores.

**Table 2**. Description of neuropsychological measures and variables included in analyses

| Domain | Subdomain | Measure | Dependent Variable | Description |
|---|---|---|---|---|
| Memory | Nonverbal Object Recognition | Pattern Recognition Memory | Number correct | Total number of items remembered; higher scores indicative of better memory |
| | | Delayed Matching to Sample | Number correct | Total number of items correct after a delay; higher scores indicative of better memory |
| | Visuospatial Memory | Paired Associates Learning | Total errors | Total number of errors, adjusted for stages not completed; lower scores indicative of better memory |
| Executive Function | Inhibitory Control | Inhibition | Total errors | Total number of errors on the Inhibition condition; lower scores indicative of better response inhibition |
| | Cognitive Fluency | Design Fluency | Number correct | Total number of correct designs across both structured and unstructured conditions; higher scores indicative of better cognitive fluency/flexibility |
| | Working Memory | Spatial Span | Span length | Maximum number of boxes remembered in a series; higher scores indicative of better spatial attention and working memory |
| | | Spatial Working Memory | Total errors | Total number of errors; lower scores indicative of better working memory |
| | Planning | Spatial Working Memory | Strategy score | Number of times a participant begins a new search with a different box; lower scores represent more efficient planning |
| Attention | Hyperactivity | Quotient ADHD System | Movements | Number of position changes; higher scores indicative of hyperactivity |
| | | | Immobility duration | Average amount of time spent sitting still (ms); lower scores indicative of hyperactivity |
| | | | Displacement | Total distance moved by the marker (m); higher scores indicative of hyperactivity |
| | | | Area | Total area covered by the marker's path (m); higher scores indicative of hyperactivity |
| | Sustained Attention | Quotient ADHD System | Accuracy | Percentage of correct responses; higher scores represent better attention |
| | | | Omission errors | Percentage of missed targets; higher scores indicative of inattention |
| | | | Response latency | Average amount of time to respond correctly (ms); higher scores indicative of inattention |
| | Impulsivity | Quotient ADHD System | Commission errors | Percentage of incorrect responses to non-targets; higher scores indicative of impulsivity |
| Motor | Fine motor dexterity | Grooved Pegboard | Dominant hand completion time | Time taken to complete task with dominant hand; more negative scores indicative better motor ability |
| | | | Non-dominant hand completion time | Time taken to complete task with non-dominant hand; more negative scores indicative better motor ability |

## Statistical Analyses

Statistical analyses were conducted using SPSS Statistics version 20.0 (IBM Corporation, 2011). An alpha level of $p < .05$ (two-tailed) was used to determine statistical significance.

**Demographic Data and Sample Characteristics**. Demographic data were analyzed using Chi-square statistics (gender, race, ethnicity, handedness, FAS diagnosis, and home placement) and independent-samples *t*-tests (age, body weight, and SES). Demographic variables were included as covariates if they were significantly correlated with the dependent variable and did not interact with either the dependent or independent variables.

**Compliance and Adverse Events**. Data on treatment adherence were analyzed using Mann-Whitney U tests owing to the non-normal distribution of compliance variables. Group comparisons on the number of participants who reported adverse and positive events were analyzed using Fisher's exact tests.

**Dietary Intake**. Data from the 24-hour dietary recall were analyzed using separate 2 (Group) x 2 (Time) analyses of variance (ANOVAs) across 30 dietary variables to assess group differences at each time point and evaluate potential confounding from changes in participants' dietary intake throughout the treatment period. Then, mean values for each nutrient were created, averaged across pre- and post-test, to obtain observed daily nutrient intake levels. One-sample *t*-tests were used to compare observed nutrient intakes to national data for children ages 5-10 years (*What We Eat in America*, NHANES 2007-2008; Agricultural Research Service - Food Surveys Research

Group, 2010). Data from the NHANES 2007-2008 were also collected using 24-hour dietary recalls and with similar collection and processing methods as the ASA24; both the NHANES and ASA24 employed the USDA's Automated Multiple-Pass Method during interview-administered 24-hour recalls, and both datasets utilized the USDA's Food and Nutrient Database for Dietary Surveys, version 4.1, making them comparable. Additionally, to evaluate whether the observed nutrient intakes were adequate based on recommended values, observed nutrient intakes were compared to the Dietary Reference Intakes (Institute of Medicine, 2006). Dietary Reference Intakes (DRIs) are dietary recommendations established for a given age range to meet or exceed the requirements of the majority of healthy individuals within that age range. For the current sample, participants' intakes were compared to DRIs for 5-8 years olds or 9-10 year olds, based on their age. The DRIs include three reference values, the Estimated Average Requirement (EAR), Recommended Dietary Allowance (RDA), and the Adequate Intake (AI). The EAR is the average daily intake level that is estimated to meet the nutrient needs of 50% of healthy individuals; the RDA is the estimated level sufficient to meet the nutrient requirements of 97-98% of healthy individuals; and the AI is the recommended average daily intake level when neither the EAR nor RDA have been established due to insufficient research. Owing to the large number of comparisons, the Holm-Bonferroni sequential procedure was used to correct for multiple tests within each category of nutrients (i.e., macronutrients, vitamins, and minerals).

**Cognitive Performance**. Data were analyzed as a 2 (Group: choline, placebo) x 2 (Time: pre-test; post-test) mixed model design with Group as a between-subjects factor and Time as a within-subjects factor. Primary outcome measures included performance

on cognitive measures of (1) memory (DMS, PRM, PAL), (2) executive function (Inhibition, Design Fluency, SWM, SSP), and (3) attention (Quotient). Each analysis was conducted two ways. First, intent-to-treat (ITT) analyses using linear mixed effects models, which allow for unbalanced designs (i.e., missing data), were conducted on all participants randomized ($n$ = 55). ITT is a strategy for the analysis of randomized controlled trials that compares participants in the groups in which they were originally randomly assigned, minimizing selection bias and preserving randomization. All participants were included in analyses according to their treatment group, as designated by the randomization procedure, regardless of adherence to the entry criteria or deviation from protocol (Hollis & Campbell, 1999). ITT analysis is the most conservative analysis, addressing pragmatic hypotheses about the clinical utility of treatment and, therefore, is the most convincing when it yields a positive result (Chene et al., 1998; Oakes et al., 1993). Second, these analyses were repeated using general linear models including only participants who completed the study ($n$ = 52). Given the possibility that poor treatment adherence may dilute a true treatment effect, completers analyses take compliance into account (Chene, et al., 1998). The completers analyses supplement ITT analyses to explore important clinical, therapeutic, and prognostic distinctions that may be otherwise lost in a small sample (Feinstein, 1991). In all analyses, age was included as a covariate if it was significantly correlated with the dependent variable and did not interact with either of the independent variables. All possible main and interaction effects were explored. Support for study hypotheses would be evidenced by a significant treatment Group x Time interaction, with simple effects analyses indicating a significant effect of Group at post-test but not pre-test. Throughout the results section, only data from participants who

completed the study, receiving assessment at both time points, are presented graphically. In the interest of determining any potential effects of the intervention on cognitive performance, Cohen's *d* effect sizes were calculated for change from pre- to post-assessment in each group on primary measures.

**Predictors of Treatment Outcomes**. Secondary analyses were conducted to examine moderators of treatment outcomes. Moderator variables of interest included intervention adherence (e.g., percent of days the treatment was taken, as reported on treatment diaries; percent of liquid consumed, as measured from treatment bottles), average daily dietary choline intake (i.e., average choline intake level from the ASA24 across pre- and post-test), and average daily total choline intake (i.e., average choline consumed in both diet and intervention). Bivariate Pearson's correlations were first performed to assess the association between predictor variables and cognitive outcomes at post-test. Correlations that were significant at $p < .05$ were considered for inclusion in subsequent hierarchical multiple linear regression analyses to assess the unique influence of each predictor on cognitive performance at post-test, after accounting for age, group, and cognitive performance at pre-test. In each regression model, cognitive performance at post-test was the dependent variable. Age (if significantly related to the dependent variable), treatment group, and cognitive performance at pre-test were entered as covariates on Step 1 (Model 1), the predictor variable was entered on Step 2 (Model 2), and the interaction term between predictor variable and Group was entered on Step 3 (Model 3). Support for study hypotheses would be evidenced by a significant positive relationship between treatment compliance and cognitive performance in only the choline group (Group x Compliance interaction) and a negative relationship between dietary

choline intake and cognitive performance (Group x Dietary Choline interaction) in Model 3.

Chapter II, in part, is currently being prepared for submission for publication of the material. Nguyen, T. T.; Risbud, R. D.; Chambers, C.; & Thomas, J. D. The dissertation author was the primary investigator and author of this material.

## III. RESULTS

### Demographic Data and Sample Characteristics

**Tables 3 and 4** present descriptive data as well as statistical analyses for the demographic characteristics and cognitive performance of the ITT sample at baseline. Treatment groups did not differ on any demographic variable: age, $t(53) = -.165$, $p = .87$, gender, $\chi^2(1) = .446$, $p = .50$, body weight percentile, $t(53) = .551$, $p = .58$, race, $\chi^2(1) = .080$, $p = .78$, ethnicity, $\chi^2(2) = 2.47$, $p = .29$, SES, $t(53) = -.599$, $p = .55$, handedness, $\chi^2(2) = 3.51$, $p = .17$, FAS diagnosis, $\chi^2(2) = .091$, $p = .96$, home placement, $\chi^2(1) = 1.88$, $p = .17$, or psychiatric comorbidity, $\chi^2(1) = .667$, $p = .41$. Moreover, the number of children from each site did not differ between groups, $\chi^2(1) = .311$, $p = .58$. At baseline, groups did not significantly differ on any outcome variable ($p$s > .05).

Additionally, as this was a multi-site study, site differences on demographic variables were assessed. Sites differed on age, $t(53) = -2.33$, $p = .02$, body weight percentile, $t(53) = -2.92$, $p = .01$, and SES, $t(53) = 2.33$, $p = .02$. Participants at the Ohio site were older (Ohio $M = 8.7$ years, San Diego $M = 7.6$ years), were at a higher body weight percentile (Ohio $M = 56.1$ percentile, San Diego $M = 30.0$ percentile), and were from lower SES (Ohio $M = 44.6$, San Diego $M = 53.0$) compared to the San Diego site. No differences were observed between study locations on gender, $\chi^2(1) = 1.74$, $p = .19$, race, $\chi^2(1) = 3.89$, $p = .14$, ethnicity, $\chi^2(2) = 1.47$, $p = .48$, handedness, $\chi^2(2) = 5.25$, $p = .07$, FAS diagnosis, $\chi^2(2) = 3.17$, $p = .21$, home placement, $\chi^2(1) = 1.81$, $p = .18$, or psychiatric comorbidity, $\chi^2(1) = .414$, $p = .52$. Further, to ensure that site did not affect the results, analyses of cognitive outcomes variables were re-run with Site (San Diego,

Ohio) as a factor. The three-way interaction was not observed to be significant for any cognitive outcome variables ($p > .05$) except for PRM and SWM strategy, the findings for which are detailed further in subsequent sections.

**Table 3**. Baseline characteristics of the ITT sample

| Variable | Choline ($n$ = 29) | Placebo ($n$ = 26) | Statistical Test |
|---|---|---|---|
| Gender [$n$ (%)] | | | $p$ = .50 |
| Males | 13 (44.8) | 14 (53.8) | |
| Females | 16 (55.2) | 12 (46.2) | |
| Age [$M$ ($SD$)] | 8.3 (1.60) | 8.2 (1.94) | $p$ = .87 |
| Age Strata [$n$ (%)] | | | $p$ = .69 |
| 5-6 years | 6 (20.7) | 8 (30.8) | |
| 7-8 years | 11 (37.9) | 9 (34.6) | |
| 9-10 years | 12 (41.4) | 9 (34.6) | |
| Body Weight [$M$ ($SD$)] | 59.8 (15.91) | 63.9 (25.43) | $p$ = .47 |
| Body Weight Percentile [$M$ ($SD$)] | 44.7 (32.94) | 49.8 (35.00) | $p$ = .58 |
| Race [$n$ (%)] | | | $p$ = .78 |
| Caucasian | 20 (69.0) | 17 (65.4) | |
| African American | 5 (17.2) | 5 (19.2) | |
| Multiracial | 4 (13.8) | 4 (15.4) | |
| Ethnicity [$n$ (%)] | | | $p$ = .29 |
| Hispanic | 2 (6.9) | 3 (11.5) | |
| Not Hispanic | 25 (86.2) | 18 (69.2) | |
| Not reported | 2 (6.9) | 5 (19.2) | |
| SES [$M$ ($SD$)] | 48.5 (12.99) | 46.4 (13.65) | $p$ = .55 |
| Handedness [$n$ (%)] | | | $p$ = .17 |
| Right | 24 (82.8) | 24 (92.3) | |
| Left | 5 (17.2) | 1 (3.8) | |
| Mixed | 0 (0.0) | 1 (3.8) | |
| FAS Diagnosis* [$n$ (%)] | | | $p$ = .96 |
| FAS | 3 (10.3) | 3 (11.5) | |
| Prenatally exposed, non-FAS | 16 (55.2) | 15 (57.7) | |
| Not diagnosed | 10 (34.5) | 8 (30.8) | |
| Home Placement [$n$ (%)] | | | $p$ = .17 |
| Biological | 2 (6.9) | 5 (19.2) | |
| Adopted | 27 (93.1) | 21 (80.8) | |
| Psychiatric Comorbidity [$n$ (%)] | | | $p$ = .41 |
| None | 12 (41.4) | 8 (30.8) | |
| At least 1 diagnosis | 17 (58.6) | 18 (69.2) | |
| Medications [$n$ (%)] | | | |
| Non-cognitive | 5 (17.2) | 6 (23.1) | $p$ = .63 |
| Cognitive | 19 (65.5) | 17 (65.4) | $p$ = .85 |
| Site [$n$ (%)] | | | $p$ = .58 |
| San Diego | 11 (37.9) | 8 (30.8) | |
| Ohio | 18 (62.1) | 18 (69.2) | |

Comparisons between treatment groups were conducted using independent-samples $t$-tests for continuous variables and chi-square test for categorical variables.
*FAS, children met three diagnostic criteria based for FAS on dysmorphological exam; Prenatally exposed non-FAS, children did not meet criteria for full FAS based on dysmorphological exam but received a diagnosis on the spectrum; Not diagnosed, children were not evaluated for a diagnosis.

**Table 4**. Baseline cognitive performance of the ITT sample

| Variable | Choline ($n$ = 29) | Placebo ($n$ = 26) | Statistical Test |
|---|---|---|---|
| DMS Total Correct | 7.7 (2.99) | 7.3 (2.78) | $p$ = .59 |
| PRM Number Correct | 17.9 (4.17) | 17.7 (4.06) | $p$ = .83 |
| PAL Total Errors | 37.7 (49.95) | 36.6 (53.14) | $p$ = .94 |
| Inhibition Total Errors | 12.2 (7.38) | 13.7 (11.07) | $p$ = .56 |
| Design Fluency Number Correct | 16.1 (7.99) | 15.9 (7.71) | $p$ = .92 |
| SSP Span Length | 3.9 (1.19) | 3.7 (1.54) | $p$ = .65 |
| SWM Total Errors | 59.6 (12.99) | 62.0 (18.81) | $p$ = .61 |
| SWM Strategy | 37.4 (2.62) | 37.2 (3.56) | $p$ = .76 |
| Movements | 5462 (2558.1) | 4806 (2623.8) | $p$ = .40 |
| Immobility Duration | 76.5 (57.43) | 96.8 (67.48) | $p$ = .28 |
| Displacement | 11.5 (7.50) | 9.0 (6.20) | $p$ = .24 |
| Area | 248 (155.3) | 364 (249.9) | $p$ = .07 |
| Accuracy | 64.8 (12.60) | 70.3 (12.69) | $p$ = .13 |
| Omission Errors | 30.0 (23.42) | 23.0 (16.98) | $p$ = .25 |
| Latency | 632 (170.8) | 632 (170.8) | $p$ = .39 |
| Commission Errors | 40.6 (20.96) | 36.2 (18.61) | $p$ = .45 |
| Grooved Pegboard: Dominant | .90 (1.52) | 1.1 (2.14) | $p$ = .74 |
| Grooved Pegboard: Nondominant | 1.6 (2.78) | 1.6 (3.37) | $p$ = .98 |

Data are presented as $M$ (SD). Comparisons between treatment groups were conducted using independent-samples $t$-tests.

## Compliance

Compliance was measured using caregiver-completed treatment diaries and measurements of liquid remaining in returned treatment bottles. Treatment diaries were

returned by 98% (*n* = 51) of participants who completed the study. According to returned treatment diaries, the average compliance rate (i.e., percent of days the treatment was taken) was 96.4% for the sample. A total of 24 (47%) children missed at least one dose throughout the treatment period. The median number of days on which the treatment was missed was 0.00, and the mean was 1.64 (*SD* = 2.59). Treatment bottles were returned by 98% (*n* = 51) of participants who completed the study. According to returned treatment bottles, the average compliance rate (i.e., percent of liquid consumed) was 95.7% for the sample. The median amount of liquid missed was 5.38 ml (equivalent to 1.02 days of treatment), and the mean was 9.31 ml (equivalent to 1.77 days of treatment; *SD* = 35.89 ml). Treatment compliance, based on treatment diaries and liquid measurements, did not differ by group (*p*s > .37).

**Adverse Events**

Data on reported adverse and positive events are presented in **Table 5**. Thirty-six (65%) children reported at least one adverse event throughout the treatment period. Groups did not differ on the number of participants with reported adverse events in any category (*p*s > .15), although the number of children who reported at least one adverse event in any category was significantly higher for the choline group compared to the placebo group (*p* = .03). There were no reported serious adverse events in either group.

**Table 5**. Number of participants who reported adverse events during the course of the study

| Adverse Event | Choline ($n$ = 29) | Placebo ($n$ = 26) | Statistical Test |
|---|---|---|---|
| General Health | 4 (13.8) | 2 (7.7) | $p$ = .67 |
| Skin | 11 (37.9) | 5 (19.2) | $p$ = .15 |
| Ear, Nose, Throat | 1 (3.4) | 0 (0.0) | $p$ = 1.00 |
| Cardiovascular | 1 (3.4) | 0 (0.0) | $p$ = 1.00 |
| Respiratory | 0 (0.0) | 1 (3.8) | $p$ = .47 |
| Gastrointestinal | 12 (41.4) | 7 (26.9) | $p$ = .40 |
| Genitourinary | 0 (0.0) | 1 (3.8) | $p$ = .47 |
| Musculoskeletal | 1 (3.4) | 1 (3.8) | $p$ = 1.00 |
| Neurologic | 3 (10.3) | 0 (0.0) | $p$ = .24 |
| Behavioral | 7 (24.1) | 4 (15.4) | $p$ = .51 |
| Allergy | 1 (3.4) | 1 (3.8) | $p$ = 1.00 |
| At least 1 adverse event in any category | 23 (79.3) | 13 (50.0) | $p$ = .03 |
| Positive Events | 9 (31.0) | 6 (23.1) | $p$ = .56 |

Data are presented as $n$ (%). Comparisons between treatment groups were conducted using Fisher's exact test.

**Dietary Intake**.

Dietary nutrient intake data was collected using 24-hour dietary recalls. After each recall, parents were asked whether food amounts reported were typical. At baseline, a majority of participants' diets were reported to be typical (78.2%; $n$ = 43), 16.4% ($n$ = 9) were reported to be less than usual, and 3.6% ($n$ = 2) were more than usual. Similar rates were observed at post-test; 75.0% ($n$ = 39) of diets were reported to be typical, 17.3% ($n$ = 9) were less than usual, and 7.7% ($n$ = 4) were more than usual.

**Table 6** reports nutrient intake data for treatment groups. Separate 2 (Group) x 2 (Time) ANOVAs on the 24-hour dietary recall data revealed no significant group differences in nutrient intake across pre- and post-test (adjusted *p*s > .05) and no significant changes in nutrient intake over time across choline and placebo groups (adjusted *p*s > .05). No significant Group x Time interaction was observed for choline or any other dietary variable (adjusted *p*s > .05).

**Table 6**. Mean dietary nutrient intakes by group at pre-test and post-test

| | Choline (*n* = 29) | | Placebo (*n* = 26) | |
|---|---|---|---|---|
| | Pre-test *M* (SD) | Post-test *M* (SD) | Pre-test *M* (SD) | Post-test *M* (SD) |
| ***Macronutrients/Energy*** | | | | |
| Energy (kcal) | 1871 (780) | 1761 (523) | 1926 (739) | 1667 (569) |
| Protein (g) | 62.3 (25.3) | 62.8 (26.4) | 68.8 (28.2) | 58.9 (31.6) |
| Carbohydrate (g) | 244 (110) | 224 (65) | 250 (115) | 222 (82) |
| Sugars (g) | 110 (55) | 99 (43) | 114 (67) | 97 (48) |
| Dietary fiber (g) | 13.2 (5.9) | 12.2 (4.1) | 15.7 (7.5) | 14.0 (6.8) |
| Total fat (g) | 74.0 (36.4) | 70.5 (28.5) | 75.2 (29.6) | 62.5 (22.8) |
| Saturated fat (g) | 25.9 (13.1) | 24.6 (9.9) | 26.2 (10.9) | 22.5 (10.9) |
| Monounsaturated fat (g) | 28.2 (14.7) | 26.4 (11.5) | 27.6 (12.1) | 22.3 (9.3) |
| Polyunsaturated fat (g) | 13.7 (8.2) | 13.6 (7.4) | 15.4 (7.5) | 12.6 (7.1) |
| Omega-3 fatty acids (g) | .018 (.024) | .046 (.133) | .030 (.036) | .034 (.118) |
| ***Vitamins*** | | | | |
| Vitamin A (µg) | 573 (369) | 596 (367) | 538 (337) | 482 (352) |
| Vitamin $B_1$ (mg) | 1.55 (.88) | 1.51 (.55) | 1.59 (.74) | 1.68 (1.28) |
| Vitamin $B_2$ (mg) | 2.00 (1.0) | 2.04 (.73) | 1.86 (.76) | 1.88 (1.31) |
| Vitamin $B_6$ (mg) | 1.63 (.92) | 1.53 (.76) | 1.51 (.58) | 1.61 (1.18) |

**Table 6**. Mean dietary nutrient intakes by group at pre-test and post-test, continued

| | Choline ($n$ = 29) | | Placebo ($n$ = 26) | |
|---|---|---|---|---|
| | Pre-test *M* (SD) | Post-test *M* (SD) | Pre-test *M* (SD) | Post-test *M* (SD) |
| Vitamin $B_{12}$ (µg) | 4.81 (2.90) | 4.48 (2.04) | 3.60 (1.95) | 4.48 (4.21) |
| Vitamin C (mg) | 75.1 (106.9) | 54.6 (49.4) | 70.7 (65.0) | 65.0 (69.8) |
| Vitamin D (µg) | 5.23 (2.87) | 5.41 (2.65) | 3.59 (2.71) | 3.86 (3.33) |
| Vitamin E (mg) | 5.63 (3.65) | 5.80 (4.70) | 6.76 (4.44) | 7.22 (7.45) |
| Vitamin K (µg) | 42.4 (27.4) | 52.8 (63.8) | 50.9 (37.6) | 44.4 (27.9) |
| Folate (DFE) (µg) | 548 (459) | 512 (279) | 432 (212) | 510 (446) |
| Choline (mg) | 224 (114) | 225 (98) | 231 (102) | 193 (109) |
| Niacin (mg) | 20.4 (10.9) | 19.5 (8.6) | 19.6 (7.6) | 19.8 (13.3) |
| ***Minerals*** | | | | |
| Calcium (mg) | 917 (465) | 932 (470) | 897 (386) | 865 (377) |
| Copper (mg) | 1.04 (.38) | 1.03 (.53) | 1.04 (.44) | .99 (.50) |
| Iron (mg) | 13.7 (7.9) | 13.2 (4.4) | 14.5 (7.9) | 14.6 (10.7) |
| Magnesium (mg) | 215 (76) | 207 (90) | 238 (104) | 217 (92) |
| Phosphorus (mg) | 1127 (486) | 1131 (384) | 1197 (472) | 1068 (487) |
| Potassium (mg) | 1859 (743) | 1941 (881) | 1965 (821) | 1873 (834) |
| Selenium (µg) | 83.6 (44.7) | 84.5 (51.6) | 95.4 (45.3) | 83.6 (62.4) |
| Zinc (mg) | 10.2 (4.4) | 9.5 (4.2) | 9.3 (3.8) | 10.4 (9.2) |

Data are presented as *M* (SD). Comparisons between treatment groups were conducted using 2x2 ANOVA.
No significant ($p < .05$) group differences or Group x Time interactions were observed after *p*-values were adjusted using the Holms-Bonferroni sequential procedure.

Nutrient intake data for the sample, compared to estimates from the NHANES and IOM Dietary Reference Intakes, are reported in **Table 7**. NHANES estimates were based on a sample size of $N$ = 1047 children ages 5-10 years. Participants did not

significantly differ on body weight (sample $M$ = 61.8, NHANES $M$ = 66.9; $p$ = .07) or total caloric intake (adjusted $p$ > .05) from the NHANES sample. Compared to the national sample, children with prenatal alcohol exposure consumed significantly lower levels of protein, omega-3 fatty acids, magnesium, potassium, zinc, vitamins C and K, niacin, and choline in their diets (adjusted $p$s < .05).

Compared to the Dietary Reference Intakes, alcohol-exposed children's diets were inadequate for several important macronutrients, vitamins, and minerals. Participants' nutrient intake levels were significantly lower than the AI for dietary fiber, potassium, omega-3 fatty acids, and choline (adjusted $p$s < .05). Additionally, vitamin K levels were significantly lower than the AI for 5-8 year-olds (adjusted $p$ = .01) but not 9-10 year-olds (adjusted $p$ > .99); conversely, calcium intake was significantly lower in 9-10 year-olds (adjusted $p$ = .02) but not 5-8 year-olds (adjusted $p$ = .96). Observed levels of vitamin E for the sample were significantly lower than the RDA (adjusted $p$s < .03) but not the EAR ($p$s > .12), suggesting that participants' diets were not quite inadequate but likely suboptimal for this vitamin. For all reported nutrients, over 50% of the sample did not meet the RDA or AI.

Although nutritional supplements were not included in these analyses, 36% of the sample ($n$ = 20) was reported by caregivers to take at least one daily supplement. Supplements included multivitamins (33%; $n$ = 18), vitamin D/calcium (6%; $n$ = 3), omega-3 fatty acids (9%; $n$ = 5), probiotic or digestive supplement (7%; $n$ = 4), zinc/magnesium/iron (2%; $n$ = 1), and lithium orotate (2%; $n$ = 1). No participant was reported to take any choline-specific supplements. Caregivers were instructed not to

make any changes to their children's dietary supplement regimen during the course of the study, and all caregivers denied any changes in supplements at the follow-up appointment.

**Table 7**. Observed dietary nutrient intakes compared to NHANES and IOM Dietary Reference Intakes

| | | | Observed Mean Nutrient Intake | | |
|---|---|---|---|---|---|
| | RDA/AI 5-8y; 9-10y | NHANES 5-10 Mean | 5-8 years (*n* = 34) | 9-10 years (*n* = 21) | Total sample (*N* = 55) |
| ***Macronutrients/Energy*** | | | | | |
| Energy (kcal) | – | 2009 | 1674 (425) | 2043 (663) | **1815 (553)**† |
| Protein (g) | 19; 19 | 74.3 | 59.1 (21.2) | 71.0 (16.8) | **63.5 (20.2)**† |
| Carbohydrate (g) | 130; 130 | 252 | 221 (68.3) | 260 (95.4) | 236 (81.2) |
| Sugars (g) | – | 120 | 101 (38.1) | 114 (59.5) | 106 (47.4) |
| Dietary fiber (g) | 25; 31m, 26f | 14.1 | **13.6 (5.5)*** | **13.9 (4.6)*** | 13.7 (5.1) |
| Total fat (g) | – | 75.5 | 64.0 (17.6) | 82.1 (30.7) | 70.9 (24.8) |
| Saturated fat (g) | – | 25.0 | 21.9 (5.9) | 29.6 (10.4) | 24.9 (8.7) |
| Monounsaturated fat (g) | – | 27.8 | 24.3 (7.7) | 29.8 (12.4) | 26.4 (10.0) |
| Polyunsaturated fat (g) | – | 16.2 | 12.5 (4.6) | 15.9 (8.5) | **13.8 (6.5)**† |
| Omega-3 fatty acids (g) | .9; 1.2m, 1.0f | .12 | **.017 (.016)*** | **.055 (.10)*** | **.031 (.065)**† |
| ***Vitamins*** | | | | | |
| Vitamin A (μg) | 400; 600 | 577 | 480 (233) | 646 (275) | 544 (260) |
| Vitamin $B_1$ (mg) | .6; .9 | 1.54 | 1.48 (.72) | 1.75 (.61) | 1.58 (.68) |
| Vitamin $B_2$ (mg) | .6; .9 | 2.10 | 1.74 (.72) | 2.27 (.68) | 1.94 (.75) |
| Vitamin $B_6$ (mg) | .6; 1 | 1.83 | 1.45 (.61) | 1.77 (.68) | 1.57 (.65) |
| Vitamin $B_{12}$ (μg) | 1.2; 1.8 | 5.15 | 3.79 (1.98) | 5.25 (2.21) | **4.34 (2.17)**† |
| Vitamin C (mg) | 25, 45 | 90.7 | 66.7 (61.2) | 66.2 (63.0) | **66.5 (61.4)**† |
| Vitamin D (μg) | 5; 5 | 5.09 | 4.27 (2.49) | 4.96 (2.08) | 4.53 (2.34) |
| Vitamin E (mg) | 7; 11 | 6.78 | **5.57 (2.78)*** | **7.31 (5.50)*** | 6.23 (4.08) |
| Vitamin K (μg) | 55; 60 | 79.2 | **40.7 (24.2)*** | 60.0 (48.0) | **48.1 (36.1)**† |

**Table 7**. Observed dietary nutrient intakes compared to NHANES and IOM Dietary Reference Intakes, continued

| | RDA/AI 5-8y; 9-10y | NHANES 5-10 Mean | Observed Mean Nutrient Intake 5-8 years (*n* = 34) | 9-10 years (*n* = 21) | Total sample (*N* = 55) |
|---|---|---|---|---|---|
| Folate (DFE) (μg) | 200; 300 | 511 | 324 (158) | 555 (270) | 499 (260) |
| Choline (mg) | 250; 375 | 295 | **207 (71.2)*** | **241 (70.7)*** | **220 (72.3)**† |
| Niacin (mg) | 8; 12 | 22.7 | 18.2 (7.0) | 22.6 (7.2) | **19.9 (7.3)**† |
| ***Minerals*** | | | | | |
| Calcium (mg) | 800; 1300 | 931 | 803 (301) | **1058 (347)*** | 900 (340) |
| Copper (mg) | .44; .7 | 1.20 | .978 (.34) | 1.10 (.41) | **1.03 (.37)**† |
| Iron (mg) | 10; 8 | 14.5 | 12.8 (5.7) | 15.6 (6.1) | 13.9 (6.0) |
| Magnesium (mg) | 130; 240 | 259 | 212 (70.7) | 230 (82.8) | **219 (75.3)**† |
| Phosphorus (mg) | 500; 1250 | 1243 | 1038 (346) | 1287 (306) | **1133 (350)**† |
| Potassium (mg) | 3800; 4500 | 2417 | **1871 (677)*** | **1996 (669)*** | **1917 (670)**† |
| Selenium (μg) | 30; 40 | 99.5 | 79.5 (37.1) | 99.0 (31.1) | **87.0 (36.0)**† |
| Zinc (mg) | 5; 8 | 11.5 | 9.05 (3.79) | 11.2 (4.29) | **9.88 (4.09)**† |

Data are presented as *M* (SD). Comparisons between sample means and reference values were conducted using one-sample *t*-tests. The Holm-Bonferroni sequential procedure was used to correct for multiple tests within each category of nutrients.
*Observed dietary intake significantly lower than Institute of Medicine RDA/AI levels, adjusted $p < .05$.
†Observed dietary intake significantly lower than *NHANES* national sample, adjusted $p < .05$.
f, female; IOM, Institute of Medicine; m, male; NHANES, National Health and Nutrition Examination Survey; y, years

## Aim 1: Memory

Demographic variable of age was significantly related to memory performance scores at both pre- and post-test and was included in all analyses as a covariate.

**Missing Data**. Differences in sample sizes across memory analyses are attributable to one participant in the placebo group who was unable to complete testing at post-test due to behavioral problems.

**Intent-to-Treat Analyses**. The effectiveness of the intervention was assessed by comparing pre- and post-test memory performance between groups. Three 2 (Group) x 2 (Time) repeated measures mixed-effects models were performed separately for DMS, PRM, and PAL variables.

Analysis of DMS number correct revealed no significant main effect of Group, $F(1,52.3) = .078$, $p = .78$, main effect of Time, $F(1,51.1) = .414$, $p = .52$, or Group x Time interaction, $F(1,51.1) = .258$, $p = .614$; Age was a significant covariate, $F(1,51.4) = 22.9$, $p < .001$. Based on the observed patterns of means and standard deviations, effect sizes across pre- and post-test were small in both the choline ($d = -.14$) and placebo ($d = .00$) groups.

For PRM number correct, analyses revealed no significant main effect of Group, $F(1,48.8) = .609$, $p = .44$, main effect of Time, $F(1,48.2) = .281$, $p = .60$, or Group x Time interaction, $F(1,48.2) = .1.07$, $p = .31$; Age was a significant covariate, $F(1,48.3) = 14.4$, $p < .001$. Effect sizes were small across pre- and post-test for the choline group ($d = .14$) and placebo group ($d = -.12$).

For PAL total errors, analyses revealed a main effect of Time, $F(1,52.2) = 5.85$, $p = .02$; both groups significantly improved at post-test compared to pre-test. No main effect of Group, $F(1,52.6) = .076$, $p = .78$, or Group x Time interaction, $F(1,52.2) = .123$, $p = .73$, was observed. Age was a significant covariate, $F(1,52.0) = 6.35$, $p = .02$. Across

pre- and post-test, effect sizes were small: $d$ = -.17 for the choline group and $d$ = -.23 for the placebo group.

**Completers Analyses**. Three 2 (Group) x 2 (Time) repeated measures ANOVAs were performed separately for DMS, PRM, and PAL variables. Mean scores of memory performance for each group at pre- and post-test are presented in **Figures 2A-C**.

For DMS number correct, PRM number correct, and PAL total errors, Age was a significant covariate [DMS: $F(1,48) = 23.9$, $p < .001$, $\eta_p^2 = .33$; PRM: $F(1,49) = 13.1$, $p = .001$, $\eta_p^2 = .21$; PAL: $F(1,48) = 6.5$, $p = .01$, $\eta_p^2 = .12$]. Analyses of all three measures of memory did not reveal any significant main effects of Group [DMS: $F(1,48) = .058$, $p = .81$, $\eta_p^2 < .01$; PRM: $F(1,49) = .752$, $p = .39$, $\eta_p^2 = .02$; PAL: $F(1,48) = .014$, $p = .91$, $\eta_p^2 < .01$] or Time [DMS: $F(1,48) = .002$, $p = .96$, , $\eta_p^2 < .01$; PRM: $F(1,49) = .141$, $p = .71$, $\eta_p^2 < .01$; PAL: $F(1,48) = .769$, $p = .39$, , $\eta_p^2 = .02$]. Moreover, no Group x Time interactions were observed [DMS: $F(1,48) = .231$, $p = .63$, $\eta_p^2 = .01$; PRM: $F(1,49) = 1.03$, $p = .32$, $\eta_p^2 = .02$; PAL: $F(1,48) = .176$, $p = .68$, $\eta_p^2 < .01$]. However, when Site was considered as a factor, the three-way interaction between Group x Time x Site was found to be significant ($p = .002$) for PRM. Follow-up of this interactive effect revealed that only within the San Diego cohort, children in the choline group showed improved memory performance at post-test ($p = .007$) that differed significantly from the placebo group ($p = .02$). **Figure 2D** depicts the three-way interaction.

For all three measures, effect sizes were small across pre- and post-test. Cohen's $d$ values were as follows: DMS choline group ($d$ = -.14) and placebo group ($d$ = .00), PRM choline group ($d$ = .03) and placebo group ($d$ = -.21), and PAL choline group ($d$ = -.17) and placebo group ($d$ = -.25).

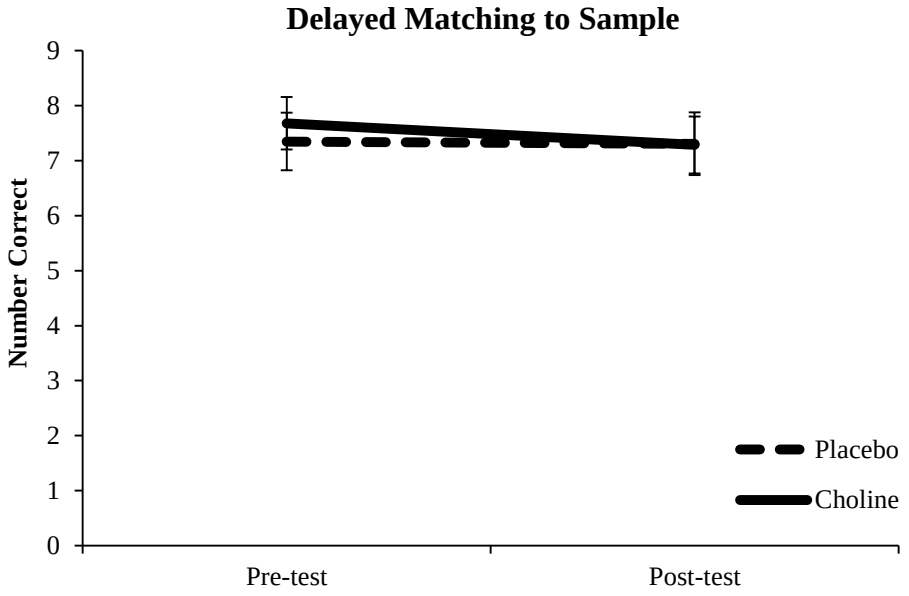


**Figure 2A**. Mean nonverbal object recognition scores from the Delayed Matching to Sample task for the choline ($n$ = 28) and placebo ($n$ = 23) groups. Data displayed are the estimated marginal means of raw scores, adjusted for age as a covariate ($M$ = 8.3 years). Higher scores are indicative of better memory.

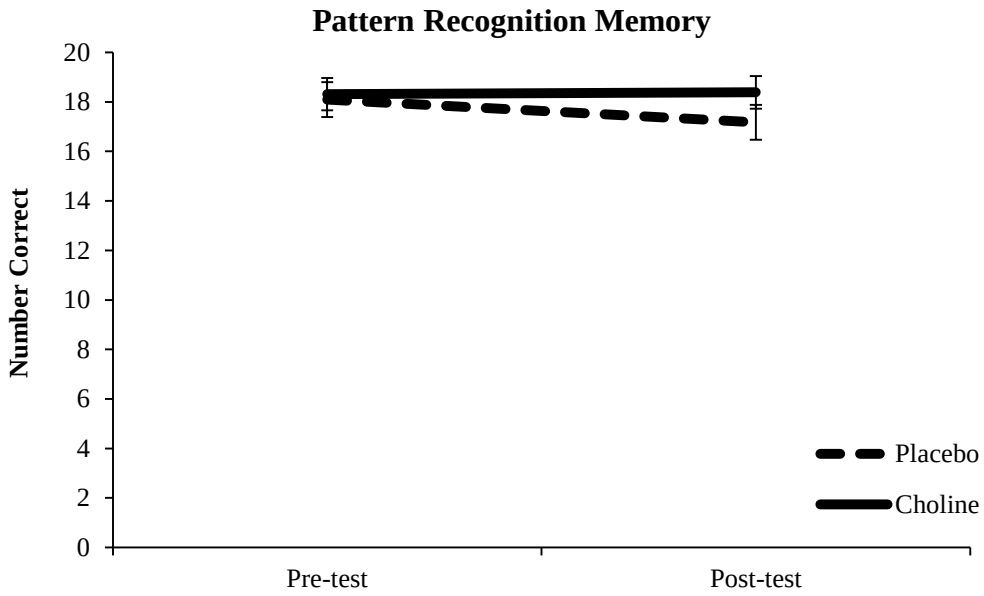


**Figure 2B**. Mean nonverbal object recognition scores from the Pattern Recognition Memory task for the choline ($n$ = 28) and placebo ($n$ = 24) groups. Data displayed are the estimated marginal means of raw scores, adjusted for age as a covariate ($M$ = 8.3 years). Higher scores are indicative of better memory.

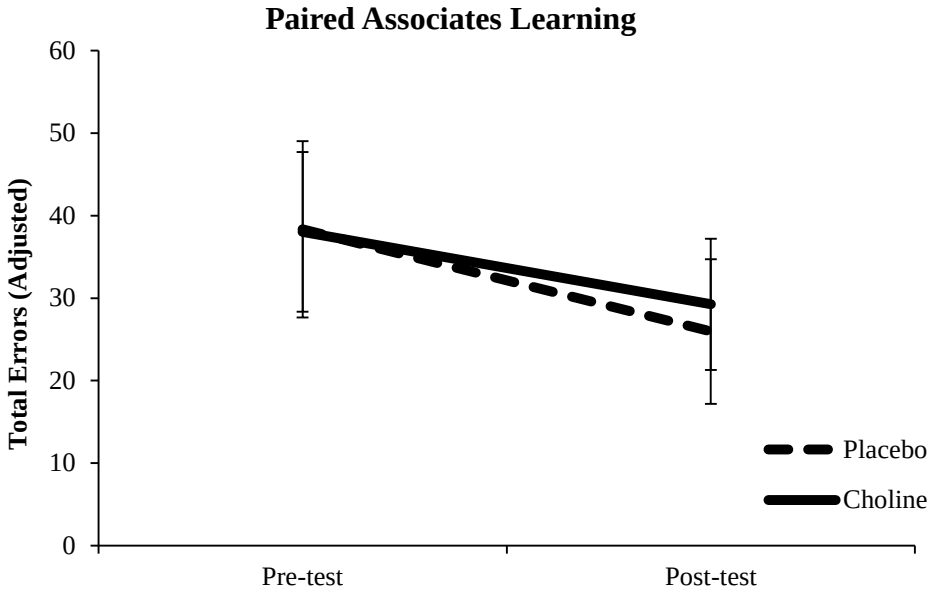


**Figure 2C**. Mean visuospatial memory scores from the Paired Associates Learning task for the choline ($n$ = 28) and placebo ($n$ = 23) groups. Data displayed are the estimated marginal means of raw scores, adjusted for age as a covariate ($M$ = 8.3 years). Lower scores are indicative of better memory.

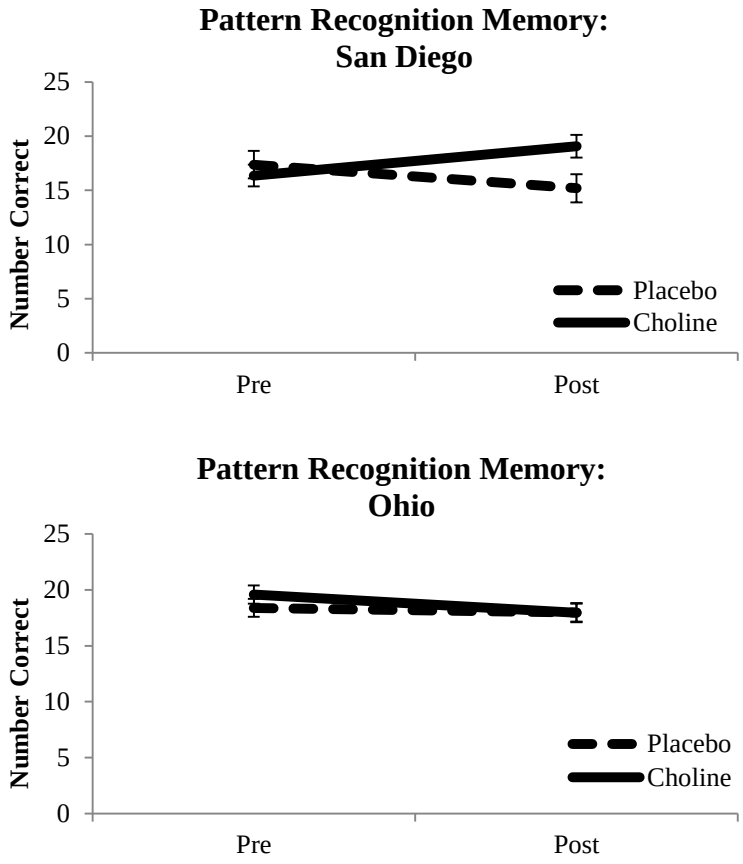


**Figure 2D**. Mean nonverbal memory scores from the Pattern Recognition Memory task for the choline and placebo groups at each Site (San Diego, Ohio).

### Aim 2: Executive Function

The demographic variable of age was significantly related to executive function performance on Inhibition, Design Fluency, SSP, and SWM total errors at both pre- and post-test and was included in these analyses as a covariate.

**Missing Data**. Three participants in the placebo group did not understand the Inhibition task and could not complete testing, and two participants in the placebo group did not complete the Design Fluency task for similar reasons. One participant in the placebo group was unable to complete the SSP and SWM tasks at post-test due to behavioral problems.

**Intention-to-Treat Analyses**. The effectiveness of the intervention was assessed by comparing pre- and post-test executive function performance between groups. Five 2 (Group) x 2 (Time) repeated measures mixed-effects models were performed separately for Inhibition, Design Fluency, SSP, SWM total errors, and SWM strategy variables. For all analyses when included, Age was a significant covariate [Inhibition: $F(1,49.3) = 22.8$, $p < .001$; Design Fluency: $F(1,19.9) = 49.0$, $p < .001$; SSP: $F(1,51.6) = 59.5$, $p < .001$; SWM total errors: $F(1,49.2) = 25.0$, $p < .001$].

Analysis of Inhibition total errors revealed a main effect of Time, $F(1,48.7) = 6.32$, $p = .02$; both groups significantly improved at post-test compared to pre-test. No significant main effect of Group, $F(1,49.7) = .275$, $p = .60$, or Group x Time interaction, $F(1,48.7) = .903$, $p = .35$, was observed. Across pre- and post-test, effect sizes were small for the choline ($d = -.19$) and placebo groups ($d = -.35$).

For Design Fluency number correct, analyses revealed a significant main effect of Time, $F(1,48.8) = 6.93$, $p = .01$; fluency performance across both groups significantly improved at post-test compared to pre-test. There was no main effect of Group, $F(1,50.1) = .760$, $p = .39$, or Group x Time interaction, $F(1,48.8) = 1.05$, $p = .31$. Effect sizes for Design Fluency were small and comparable between the choline group ($d = .28$) and placebo group ($d = .21$).

Analysis of SSP span length revealed no significant main effect of Group, $F(1,52.4) = .004$, $p = .95$, Time, $F(1,51.0) = .003$, $p = .96$, or Group x Time interaction, $F(1,51.0) = .605$, $p = .44$. Across pre- and post-test, effect sizes were very small: $d = -.07$ for the choline group and $d = .10$ for the placebo group.

For SWM total errors, there was a significant main effect of Time, $F(1,48.3) = 4.27$, $p = .04$; across both groups, SWM errors significantly declined at post-test compared to pre-test. There was no main effect of Group, $F(1,50.0) = .000$, $p = .99$, or Group x Time interaction, $F(1,48.4) = 1.02$, $p = .31$. Effect sizes were small across pre- and post-test for the choline group ($d = .07$) and placebo group (pre: $d = -.37$).

For SWM strategy, analyses did not reveal a significant main effect of Group, $F(1,51.4) = 1.68$, $p = .20$, Time, $F(1,51.0) = .303$, $p = .59$, or Group x Time interaction, $F(1,51.0) = 1.03$, $p = .32$. Across pre- and post-test, effect sizes were small in the choline group ($d = .36$) and minimal in the placebo group ($d = -.08$).

**Completers Analyses**. Five 2 (Group) x 2 (Time) repeated measures ANOVAs were performed separately for Inhibition, Design Fluency, SSP, SWM total errors, and SWM strategy variables. Again, Age was a significant covariate for Inhibition, $F(1,46) = 22.9$, $p < .001$, $\eta_p^2 = .33$, Design Fluency, $F(1,47) = 47.0$, $p < .001$, $\eta_p^2 = .50$, SSP, $F(1,48) = 57.6$, $p < .001$, $\eta_p^2 = .55$, and SWM total errors, $F(1,48) = 25.5$, $p < .001$, $\eta_p^2 = .35$. Mean scores of executive function performance for each group at pre- and post-test are presented in **Figures 3A-E**.

For completers, no significant effects were observed across any measure of executive function for Group [Inhibition: $F(1,46) = .396$, $p = .53$, $\eta_p^2 = .01$; Design Fluency: $F(1,47) = .486$, $p = .49$, $\eta_p^2 = .01$; SSP: $F(1,48) = .007$, $p = .93$, $\eta_p^2 < .01$; SWM total errors: $F(1,48) = .122$, $p = .73$, $\eta_p^2 < .01$; SWM strategy: $F(1,49) = .875$, $p = .35$, $\eta_p^2 = .02$], Time [Inhibition: Time, $F(1,46) = .485$, $p = .49$, $\eta_p^2 = .01$; Design Fluency: $F(1,47) = .294$, $p = .59$, , $\eta_p^2 < .01$; SSP: $F(1,48) = 1.63$, $p = .21$, , $\eta_p^2 = .03$; SWM total errors: $F(1,48) = .474$, $p = .49$, , $\eta_p^2 < .01$; SWM strategy: $F(1,49) = .102$, $p = .75$, $\eta_p^2$

< .01] or Group x Time interaction [Inhibition: $F(1,46) = .999$, $p = .32$, $\eta_p^2 = .02$; Design Fluency: $F(1,47) = 1.33$, $p = .26$, $\eta_p^2 = .03$; SSP: $F(1,48) = .488$, $p = .49$, $\eta_p^2 = .01$; SWM total errors: $F(1,48) = .736$, $p = .40$, $\eta_p^2 = .02$; SWM strategy: $F(1,49) = 1.72$, $p = .20$, $\eta_p^2 = .03$]. When Site was considered as a factor, the three-way interaction between Group x Time x Site was found to be significant for SWM Strategy ($p = .04$). However, follow-up of this interactive effect did not reveal significant changes from pre- to post-test for either group, although within the San Diego cohort, the choline group performed worse at post-test compared to the placebo group ($p = .03$). **Figure 3F** depicts the three-way interaction among Group, Time, and Site for SWM strategy.

Based on the observed patterns of means between pre- and post- tests, Cohen's *d* values of effect sizes were generally small across all measures for both groups: Inhibition choline group ($d = -.21$) and placebo group ($d = -.35$), Design Fluency choline group ($d = .25$) and placebo group ($d = .12$), SSP choline group ($d = -.07$) and placebo group ($d = .06$), SWM total errors choline group ($d = -.12$) and placebo group ($d = -.25$), and SWM strategy choline group ($d = .36$) and placebo group ($d = -.16$).

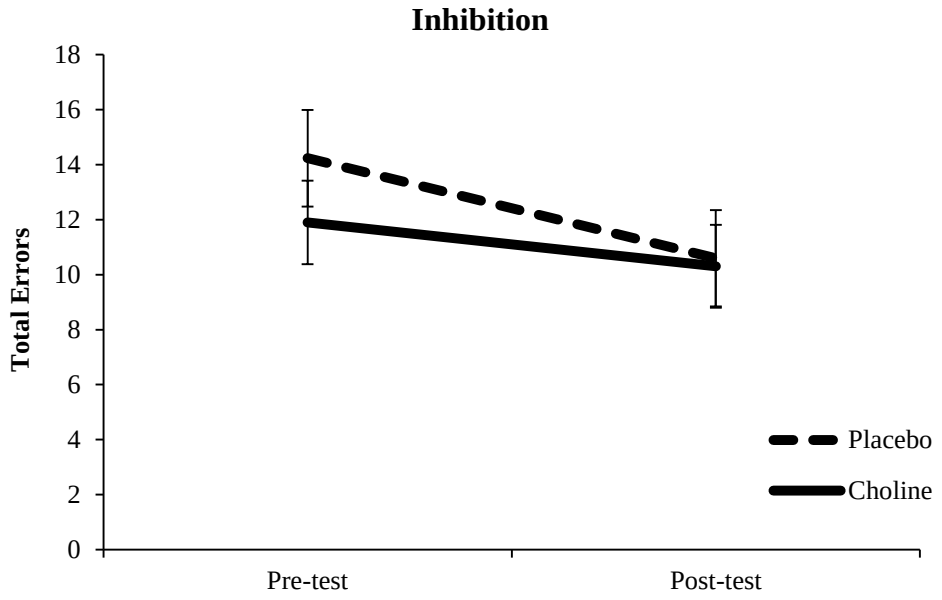


**Figure 3A**. Mean response inhibition scores from the Inhibition task (Inhibition condition) for the choline ($n$ = 28) and placebo ($n$ = 21) groups. Data displayed are the estimated marginal means of raw scores, adjusted for age as a covariate ($M$ = 8.5 years). Lower scores are indicative of better response inhibition.

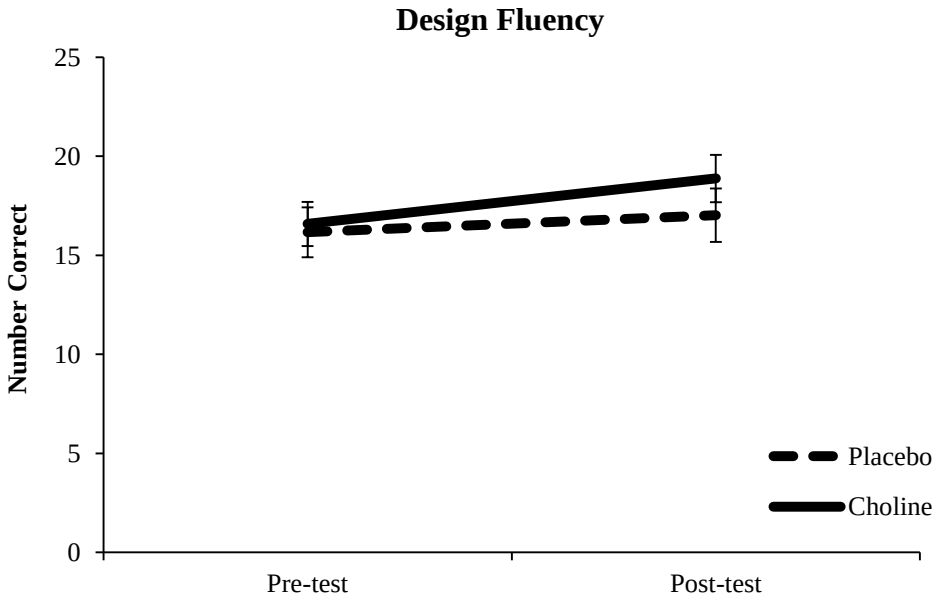


**Figure 3B**. Mean cognitive fluency scores from the Design Fluency task for the choline ($n$ = 28) and placebo ($n$ = 22) groups. Data displayed are the estimated marginal means of raw scores, adjusted for age as a covariate ($M$ = 8.5 years). Higher scores are indicative of better cognitive fluency.

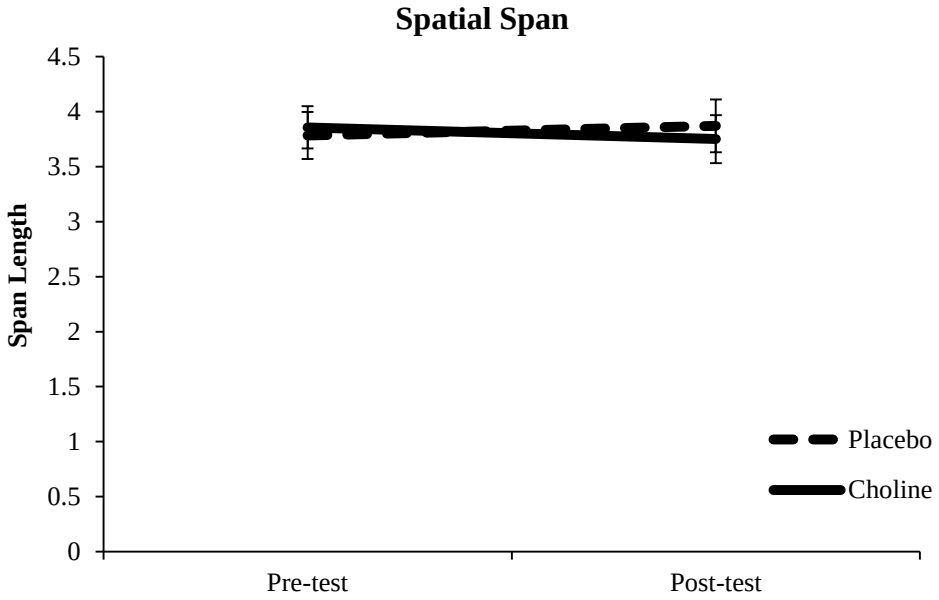


**Figure 3C**. Mean visuospatial attention and working memory scores from the Spatial Span task for the choline ($n$ = 28) and placebo ($n$ = 23) groups. Data displayed are the estimated marginal means of raw scores, adjusted for age as a covariate ($M$ = 8.3 years). Higher scores are indicative of better spatial attention and working memory.

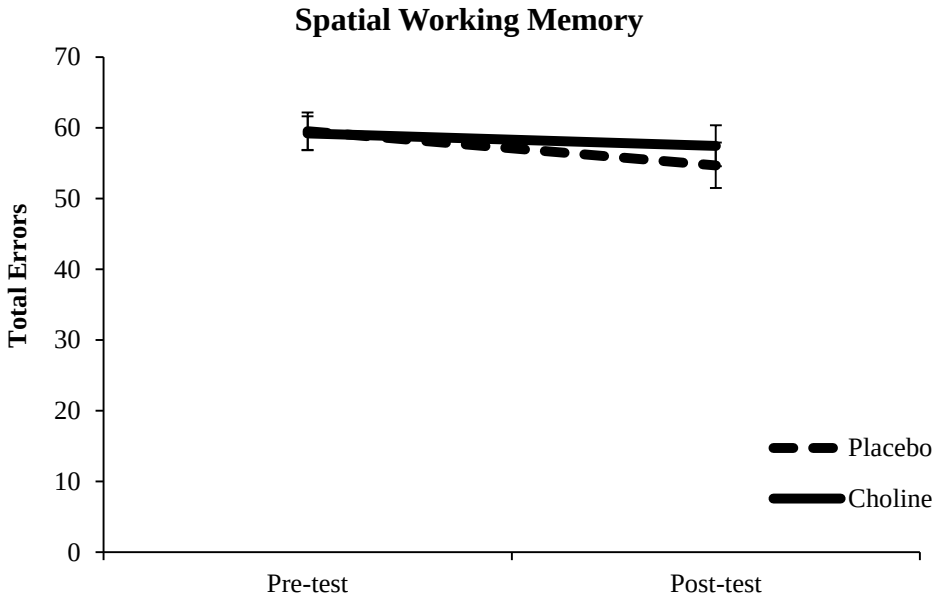


**Figure 3D**. Mean visuospatial working memory scores from the Spatial Working Memory task for the choline ($n$ = 28) and placebo ($n$ = 23) groups. Data displayed are the estimated marginal means of raw scores, adjusted for age as a covariate ($M$ = 8.3 years). Lower scores are indicative of better working memory.

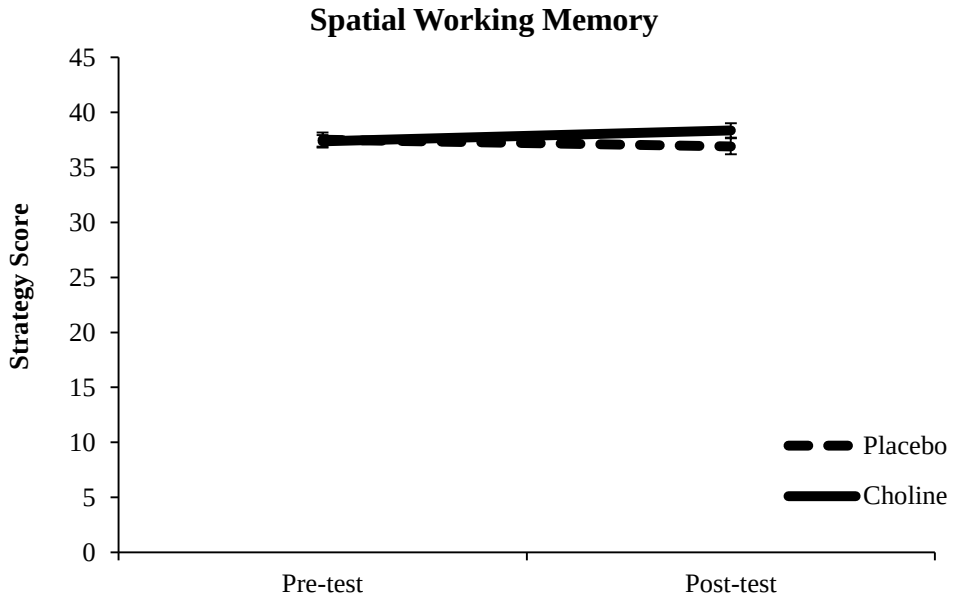


**Figure 3E**. Mean planning scores from the Spatial Working Memory task for the choline ($n$ = 28) and placebo ($n$ = 23) groups. Data displayed are the estimated marginal means of raw scores. Lower scores represent more efficient planning.

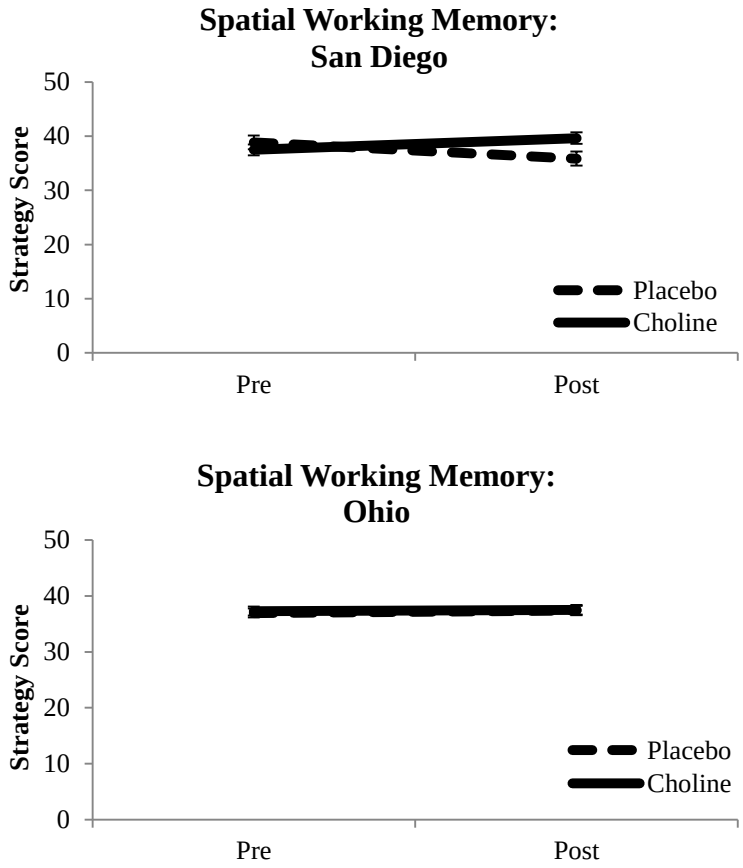


**Figure 3F**. Mean planning scores from the Spatial Working Memory task for the choline and placebo groups at each Site (San Diego, Ohio).

## Aim 3: Attention

Four measures of hyperactivity (Movements, Immobility Duration, Displacement, Area), 3 measures of inattention (Accuracy, Omission Errors, Latency), and 1 measure of impulsivity (Commission Errors) from the Quotient were assessed in Aim 3. The demographic variable of age was significantly related to Movements, Immobility Duration, Displacement, Area, Accuracy, Omission Errors, and Latency at both pre- and post-test and was included in these analyses as a covariate.

**Missing Data**. Data on inattention and impulsivity measures were missing for four participants in the placebo group and five participants in the choline group. Four children could not complete the task due to difficulties with task comprehension or behavioral problems that precluded testing, two children could not complete the task due to technical problems related to the Quotient system, and two children had scores that were invalid due to medication changes. Data on hyperactivity measures were missing for five participants in the placebo group and ten participants in the choline group for the same aforementioned reasons listed above; moreover, an additional six children did not have sufficient motor data collected due to movement outside the range of the motion tracking system.

**Intent-to-Treat Analyses**. The effectiveness of the intervention was assessed by comparing pre- and post-test attention and activity performance between groups. Attention and motor activity measures of the Quotient ADHD System were submitted to a 2 (Group) x 2 (Time) repeated measures mixed-effects models; each variable was tested separately.

***Hyperactivity***. For all measures of hyperactivity, Age was a significant covariate [Movements: $F(1,46.3) = 12.5$, $p = .001$; Immobility Duration: $F(1,48.3) = 12.4$, $p < .001$; Displacement: $F(1,44.0) = 9.36$, $p = .004$; Area: $F(1,40.1) = 6.89$, $p = .01$]. Analyses revealed no significant main or interaction effects for any measure of hyperactivity, including Movement [Group: $F(1,46.0) = 1.70$, $p = .20$; Time: $F(1,39.4) = .213$, $p = .65$; Group x Time: $F(1,39.4) = 1.60$, $p = .21$], Immobility Duration [Group: $F(1,48.0) = 2.42$, $p = .13$; Time: $F(1,42.4) = .031$, $p = .86$; Group x Time: $F(1,42.4) = 1.28$, $p = .27$], Displacement [Group: $F(1,43.8) = 2.43$, $p = .13$; Time: $F(1,37.2) = .00$, $p$

> .99; Group x Time: $F(1,37.2) = .99$, $p = .33$], or Area [Group: $F(1,39.8) = 3.66$, $p = .06$; Time: $F(1,33.9) = .128$, $p = .72$; Group x Time: $F(1,33.9) = .013$, $p = .91$].

Cohen's *d* values based on the observed pattern of means across pre-test and post-test, suggested small effect sizes for Movements [choline group: $d = .20$; placebo group: $d = -.12$], Immobility Duration [choline group: $d = -.15$; placebo group: $d = .18$], Displacement [choline group: $d = .04$; placebo group: $d = -.15$], and Area [choline group: $d = -.18$; placebo group: $d = -.06$].

***Inattention***. Age was a significant covariate for all measures of inattention [Accuracy: $F(1,45.1) = 11.2$, $p = .002$; Omission Errors: $F(1,44.7) = 7.15$, $p = .01$; Latency: $F(1,44.7) = 10.4$, $p = .002$]. Similarly, no significant main or interaction effects were observed for any measure of inattention, including Accuracy [Group: $F(1,45.7) = 1.40$, $p = .24$; Time: $F(1,41.9) = .002$, $p = .97$; Group x Time: $F(1,41.9) = .127$, $p = .72$], Omission Errors [Group: $F(1,45.2) = .280$, $p = .60$; Time: $F(1,41.1) = .660$, $p = .42$; Group x Time: $F(1,41.1) = 1.41$, $p = .24$], or Latency [Group, $F(1,45.5) = .162$, $p = .69$, Time, $F(1,42.7) = .091$, $p = .77$; Group x Time: $F(1,42.6) = .121$, $p = .74$].

Across pre- and post-test, effect sizes were very small and not suggestive of improvement in either group: Accuracy [choline group: $d = -.07$; placebo group: $d = .04$], Omission Errors [choline group: $d = -.02$; placebo group: $d = .05$], and Latency [choline group: $d = .04$; placebo group: $d = .08$].

***Impulsivity***. Analysis of Commission Errors revealed no significant main effect of Group, $F(1,) = .004$, $p = .95$, Time, $F(1,) = .003$, $p = .96$, or Group x Time interaction, $F(1,) = .605$, $p = .44$. Across pre- and post-test, effect sizes were small in the choline group ($d = -.07$) and placebo group ($d = -.09$).

**Completers Analyses**. Separate 2 (Group) x 2 (Time) repeated measures ANOVAs were performed for attention and motor activity measures of the Quotient ADHD System. Mean scores of attention performance for each group at pre- and post-test are presented in **Figures 4A-D** (hyperactivity), **Figures 5A-C** (inattention), and **Figure 6** (impulsivity).

***Hyperactivity***. Age was a significant covariate for all measures of hyperactivity [Movements: $F(1,34) = 12.5$, $p = .001$, $\eta_p^2 = .27$; Immobility Duration: $F(1,34) = 11.0$, $p = .002$, $\eta_p^2 = .24$; Displacement: $F(1,34) = 9.39$, $p = .004$, $\eta_p^2 = .22$; Area: $F(1,34) = 8.51$, $p = .006$, $\eta_p^2 = .20$]. Consistent with ITT analyses, completers analyses did not reveal any significant main or interaction effects for Movements [Group: $F(1,34) = .707$, $p = .41$, $\eta_p^2 = .02$; Time: $F(1,34) = .551$, $p = .46$, $\eta_p^2 = .02$; Group x Time: $F(1,34) = 1.24$, $p = .27$, $\eta_p^2 = .04$], Immobility Duration [Group: $F(1,34) = 1.36$, $p = .25$, $\eta_p^2 = .04$; Time: $F(1,34) = .075$, $p = .79$, $\eta_p^2 < .01$; Group x Time: $F(1,34) = .947$, $p = .34$, $\eta_p^2 = .03$], Displacement [Group: $F(1,34) = 1.00$, $p = .32$, $\eta_p^2 = .03$; Time: $F(1,34) = .751$, $p = .39$, $\eta_p^2 = .02$; Group x Time: $F(1,34) = 1.14$, $p = .29$, $\eta_p^2 = .03$], or Area [Group: $F(1,34) = 1.71$, $p = .20$, $\eta_p^2 = .05$; Time: $F(1,34) = .403$, $p = .53$, $\eta_p^2 = .01$; Group x Time: $F(1,34) = .022$, $p = .88$, $\eta_p^2 < .01$].

Cohen's *d* values across pre-test and post-test were small for both groups: Movements [choline group: $d = .23$; placebo group: $d = -.08$], Immobility Duration [choline group: $d = -.13$; placebo group: $d = .13$], Displacement [choline group: $d = .18$; placebo group: $d = -.13$], and Area [choline group: $d = .05$; placebo group: $d = -.03$].

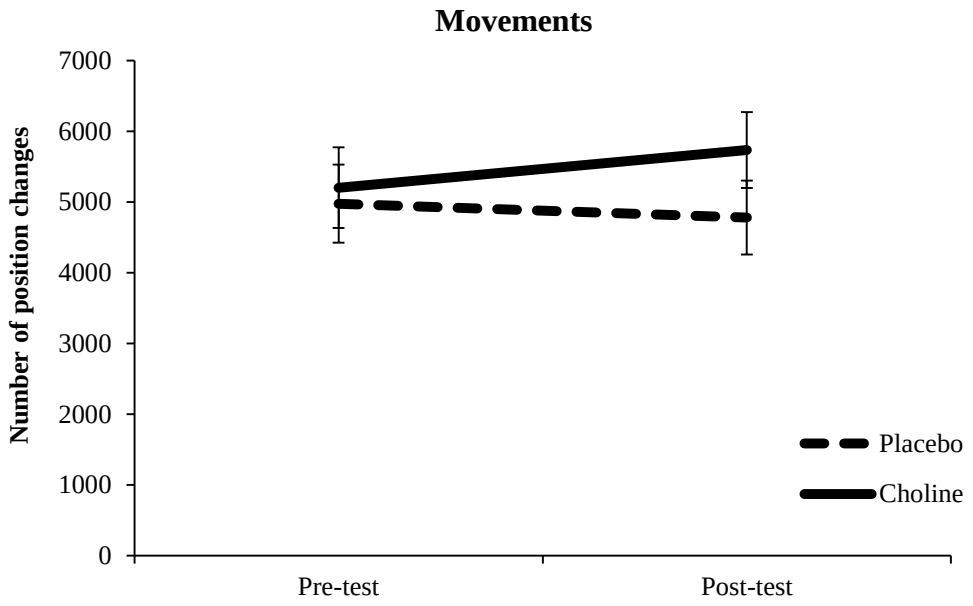


**Figure 4A**. Mean movement scores from the Quotient ADHD System for the choline ($n$ = 18) and placebo ($n$ = 19) groups. Data displayed are the estimated marginal means of raw scores, adjusted for age as a covariate ($M$ = 8.7 years). Lower scores are indicative of less hyperactivity.

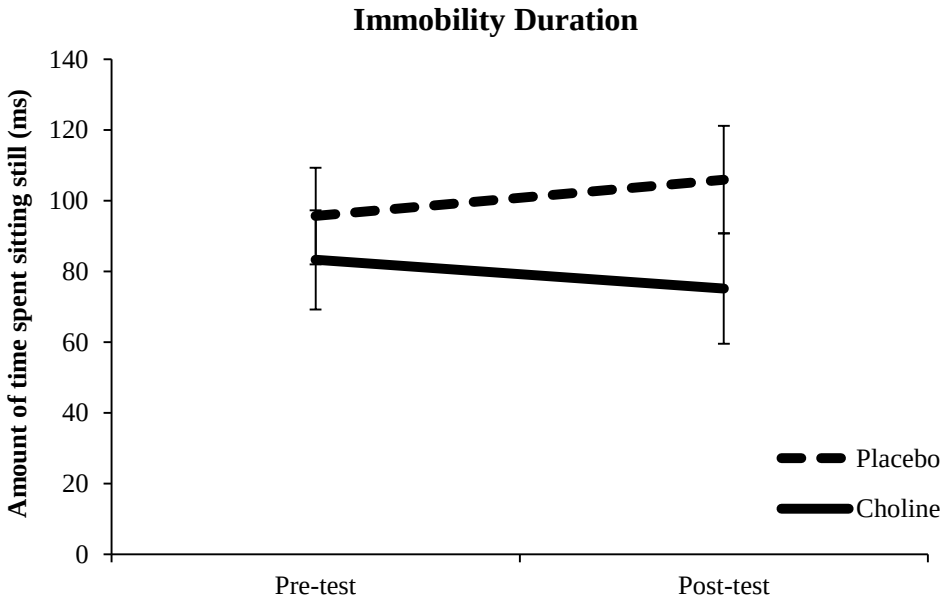


**Figure 4B**. Mean immobility duration scores from the Quotient ADHD System for the choline ($n$ = 18) and placebo ($n$ = 19) groups. Data displayed are the estimated marginal means of raw scores, adjusted for age as a covariate ($M$ = 8.7 years). Higher scores are indicative of less hyperactivity.

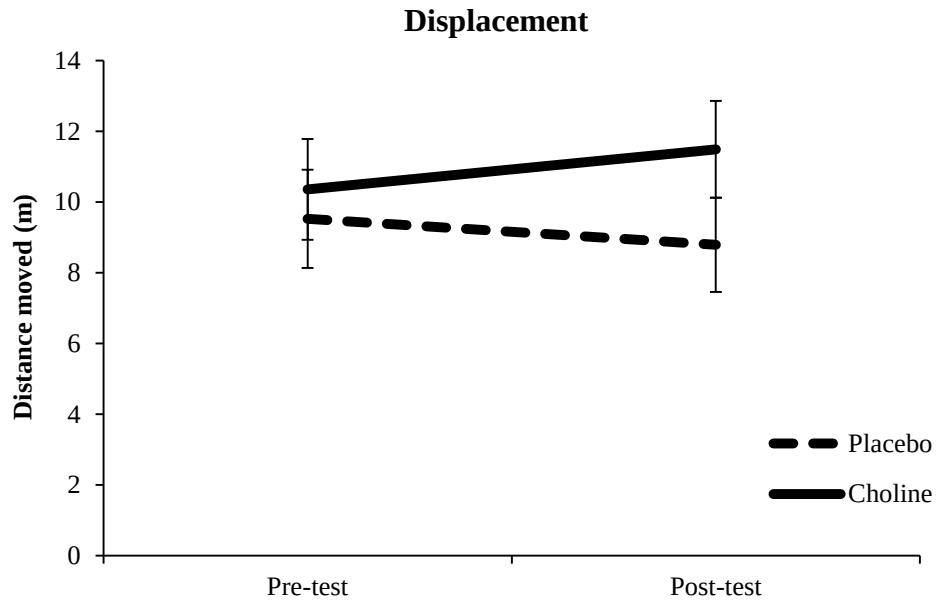


**Figure 4C**. Mean displacement scores from the Quotient ADHD System for the choline ($n$ = 18) and placebo ($n$ = 19) groups. Data displayed are the estimated marginal means of raw scores, adjusted for age as a covariate ($M$ = 8.7 years). Lower scores are indicative of less hyperactivity.

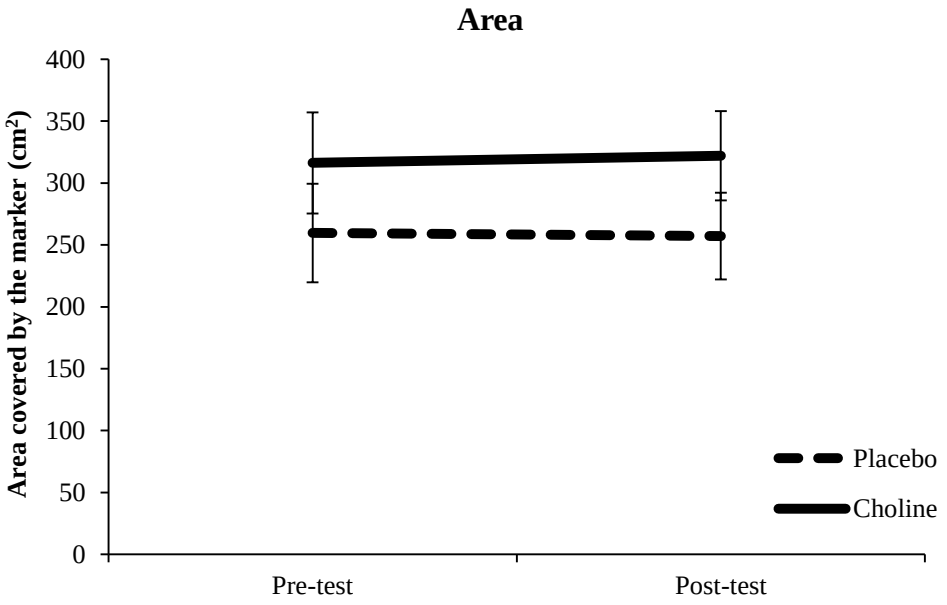


**Figure 4D**. Mean area of movement scores from the Quotient ADHD System for the choline ($n$ = 18) and placebo ($n$ = 19) groups. Data displayed are the estimated marginal means of raw scores, adjusted for age as a covariate ($M$ = 8.7 years). Lower scores are indicative of less hyperactivity.

***Inattention***. Age was a significant covariate for Accuracy, $F(1,40) = 13.0$, $p = .001$, $\eta_p^2 = .25$, and Latency, $F(1,40) = 8.52$, $p = .006$, $\eta_p^2 = .18$; for Omission Errors, Age interacted with the independent variable of Group, making it inappropriate as a covariate, and it was excluded from the model. No significant effects were found across any measure of inattention: Accuracy [Group: $F(1,40) = 1.04$, $p = .31$, $\eta_p^2 = .03$; Time: $F(1,40) = .275$, $p = .60$, $\eta_p^2 < .01$; Group x Time: $F(1,40) = .048$, $p = .83$, $\eta_p^2 < .01$], Omission Errors [Group: $F(1,41) = 2.30$, $p = .14$, $\eta_p^2 = .05$; Time: $F(1,41) = .961$, $p = .33$, $\eta_p^2 = .02$; Group x Time: $F(1,41) = 1.87$, $p = .18$, $\eta_p^2 = .04$], or Latency [Group: $F(1,40) = .288$, $p = .60$, $\eta_p^2 < .01$; Time: $F(1,40) = .319$, $p = .58$, , $\eta_p^2 < .01$; Group x Time: $F(1,40) = .251$, $p = .62$, $\eta_p^2 < .01$].

Across pre- and post-test, effect sizes were small for both groups: Accuracy [choline group: $d = .03$; placebo group: $d = -.05$], Omission Errors [choline group: $d = -.03$; placebo group: $d = .37$], and Latency [choline group: $d = -.03$; placebo group: $d = .09$]

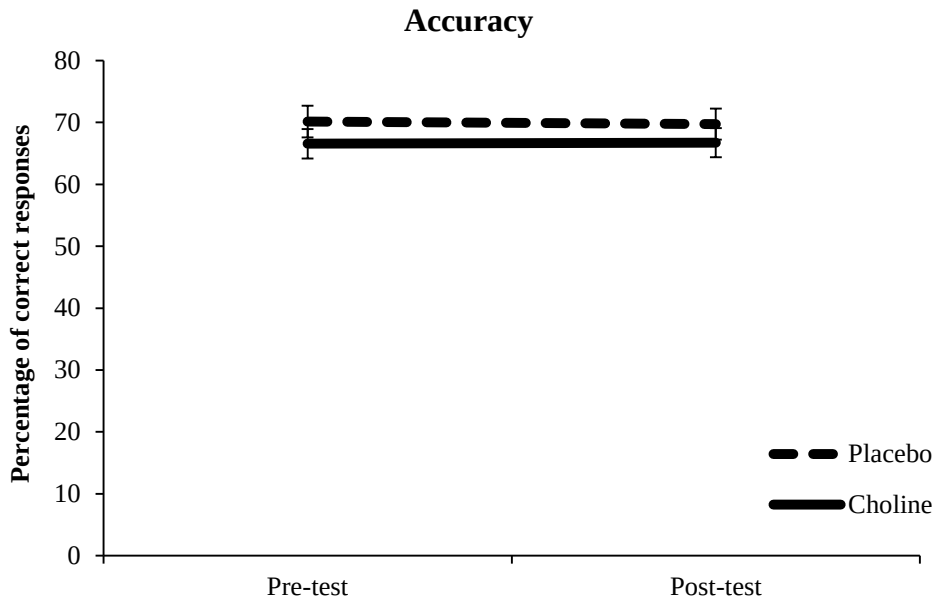


**Figure 5A**. Mean accuracy scores from the Quotient ADHD System for the choline ($n$ = 23) and placebo ($n$ = 20) groups. Data displayed are the estimated marginal means of raw scores, adjusted for age as a covariate ($M$ = 8.4 years). Lower scores are indicative of greater inattention.

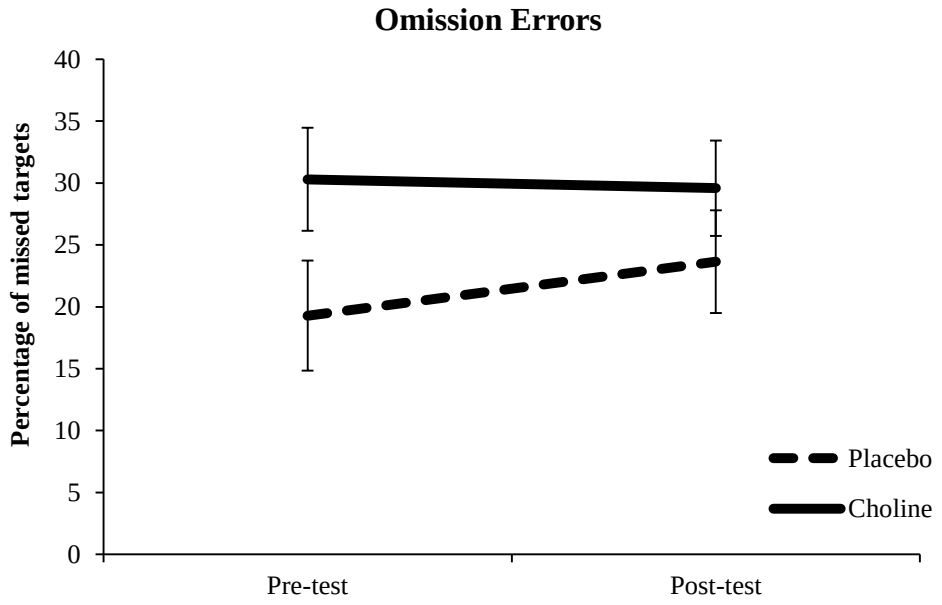


**Figure 5B**. Mean omission error scores from the Quotient ADHD System for the choline ($n$ = 23) and placebo ($n$ = 20) groups. Data displayed are the estimated marginal means of raw scores. Higher scores are indicative of greater inattention.

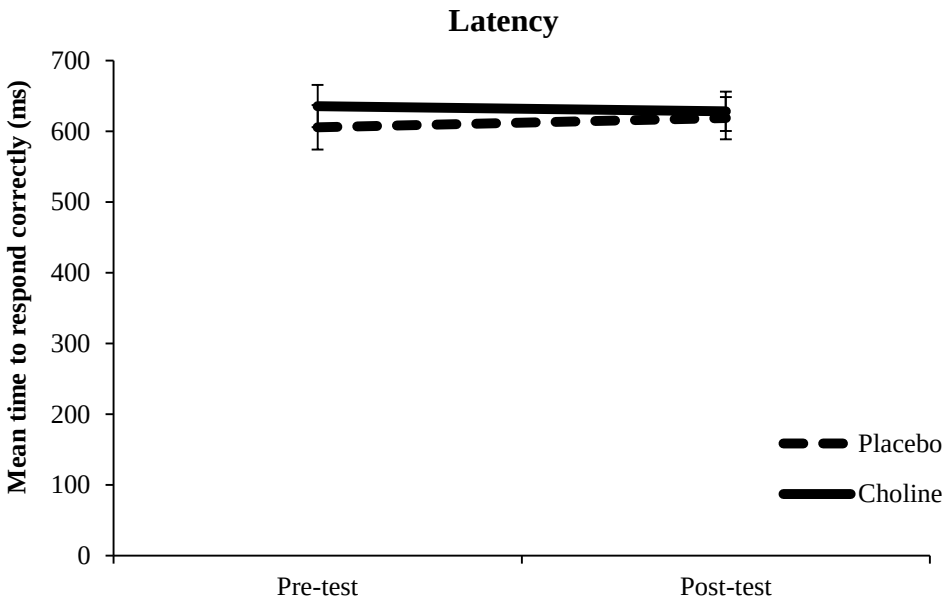


**Figure 5C**. Mean latency scores from the Quotient ADHD System for the choline ($n$ = 23) and placebo ($n$ = 20) groups. Data displayed are the estimated marginal means of raw scores, adjusted for age as a covariate ($M$ = 8.4 years). Higher scores are indicative of greater inattention.

***Impulsivity***. For Commission Errors, results revealed no significant main effect of Group, $F(1,41) = .220$, $p = .64$, $\eta_p^2 < .01$, Time, $F(1,41) = .379$, $p = .54$, , $\eta_p^2 < .01$, or Group x Time interaction, $F(1,41) = .422$, $p = .52$, $\eta_p^2 = .01$. Across pre- and post-test, effect sizes were small in both the choline ($d$ = .00) and placebo groups ($d$ = -.17).

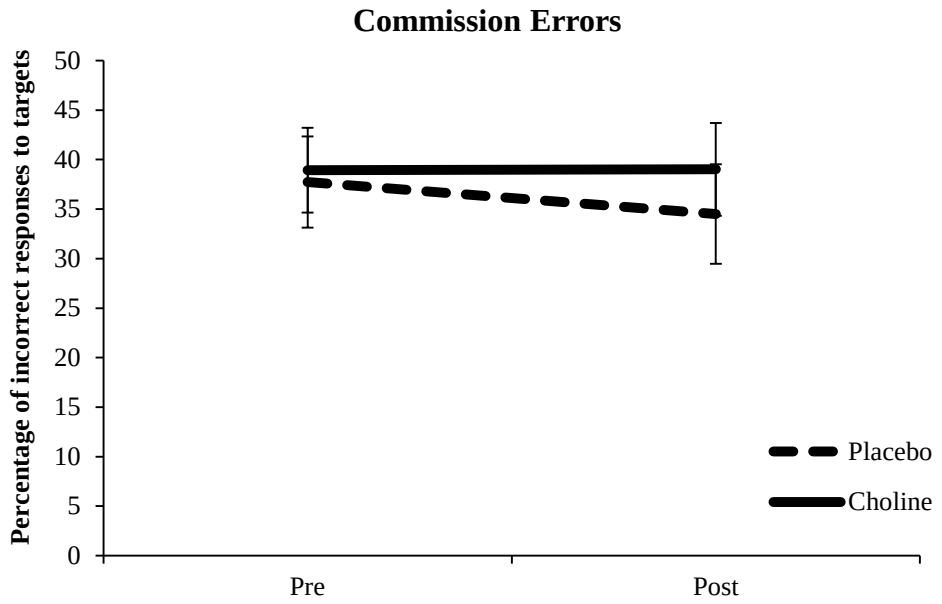


**Figure 6**. Mean commission error scores from the Quotient ADHD System for the choline ($n$ = 23) and placebo ($n$ = 20) groups. Data displayed are the estimated marginal means of raw scores. Higher scores are indicative of greater impulsivity.

### Aim 4: Motor

No demographic variables were significantly associated with motor performance; therefore, analyses were continued without covariates.

**Missing Data**. Grooved Pegboard data for the dominant hand were missing from 3 participants (1 choline, 2 placebo) due to physical limitations (e.g., broken arm), behavioral problems, or difficulty with task comprehension that precluded completion. Data for the nondominant hand was missing from 5 participants (2 choline, 3 placebo) for similar reasons.

**Intent-to-Treat Analyses**. The effectiveness of the intervention was assessed by comparing pre- and post-test motor performance between groups. Two 2 (Group) x 2

(Time) repeated measures mixed-effects models were performed separately for Grooved Pegboard dominant and non-dominant hand scores.

Analysis of Grooved Pegboard dominant hand completion time revealed a significant main effect of Time, $F(1,45.2) = 7.01$, $p = .01$. Motor performance across both groups improved at post-test. No main effect of Group, $F(1,49.6) = .106$, $p = .75$ or Group x Time interaction, $F(1,45.2) = .010$, $p = .92$, was observed. No significant main or interaction effects were observed for Grooved Pegboard non-dominant hand completion time [Group: $F(1,49.2) = .197$, $p = .67$; Time: $F(1,45.9) = 3.599$, $p = .06$; Group x Time: $F(1,45.9) = .177$, $p = .68$]. Across pre- and post-test, effect sizes were small: dominant hand choline group ($d = -.21$) and placebo group ($d = -.39$), nondominant hand choline group ($d = -.15$) and placebo group ($d = -.24$).

**Completers Analyses**. Separate 2 (Group) x 2 (Time) repeated measures ANOVAs were performed for Grooved Pegboard variables. Mean scores of motor performance for each group at pre- and post-test are presented in **Figure 7**.

For both dominant and nondominant hands, analysis of Grooved Pegboard completion time revealed a significant main effect of Time [dominant: $F(1,47) = 6.25$, $p = .02$, , $\eta_p^2 = .12$; nondominant: $F(1,45) = 4.26$, $p = .05$, , $\eta_p^2 = .09$]. Motor performance across groups improved at post-test compared to pre-test. No significant main effect of Group [dominant: $F(1,47) = .284$, $p = .60$, $\eta_p^2 < .01$; nondominant: $F(1,45) = .034$, $p = .86$, $\eta_p^2 < .01$] or Group x Time interaction [dominant: $F(1,47) = .014$, $p = .91$, $\eta_p^2 < .01$; nondominant: $F(1,45) = .200$, $p = .66$, $\eta_p^2 < .01$] was observed. Across pre- and post-test, effect sizes were small: dominant hand choline group ($d = -.23$) and placebo

group (*d* = .25) and nondominant hand choline group (*d* = -.21) and placebo group (*d* = -.30).

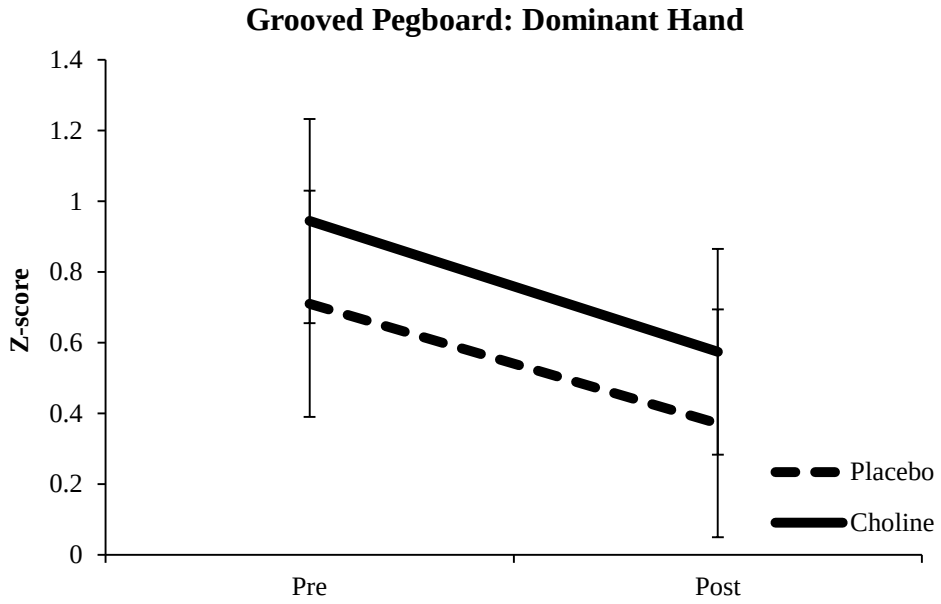


**Figure 7A**. Mean fine motor coordination scores from the dominant hand condition of the Grooved Pegboard task for the choline (*n* = 27) and placebo (*n* = 22) groups. Data displayed are the estimated marginal means of Z-scores. Lower scores are indicative of better fine-motor dexterity.

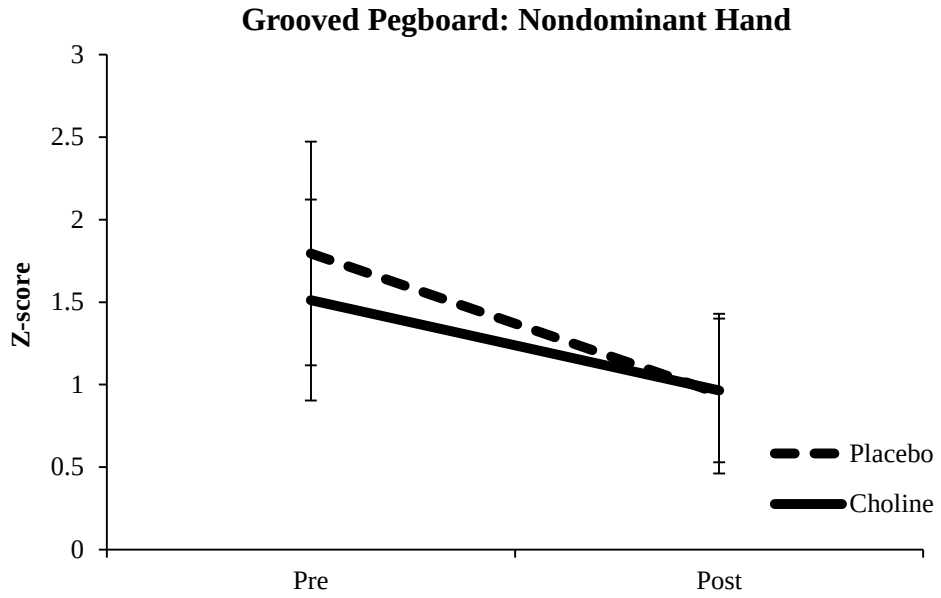


**Figure 7B**. Mean fine motor coordination scores from the non-dominant hand condition of the Grooved Pegboard task for the choline ($n$ = 26) and placebo ($n$ = 21) groups. Data displayed are the estimated marginal means of Z-scores. Lower scores are indicative of better fine-motor dexterity.

## The Contribution of Age

Age was significantly related with most dependent variables and was included a covariate when appropriate. However, given the possibility that children in various ages groups might differentially respond to treatment, primary data analyses were repeated with Age Group (e.g., 5-6 years, 7-8 years, 9-10 years) as a between-subjects factor to investigate if a therapeutic window, during which choline supplementation may be most effective, exists. Such findings might be overshadowed by adjusting results for age, alone. A Group x Time x Age Group interaction would suggest that a treatment effect varies by age.

No significant Group x Time x Age Group interaction were observed for any cognitive outcome variable except DMS, $F(2,45) = 3.97$, $p = .03$, $\eta_p^2 = .15$. However,

further examination of this interaction revealed spurious relationships that did not appear to be driven by true effects. **Figure 8** depicts the three-way interaction among Group, Time, and Age Group.

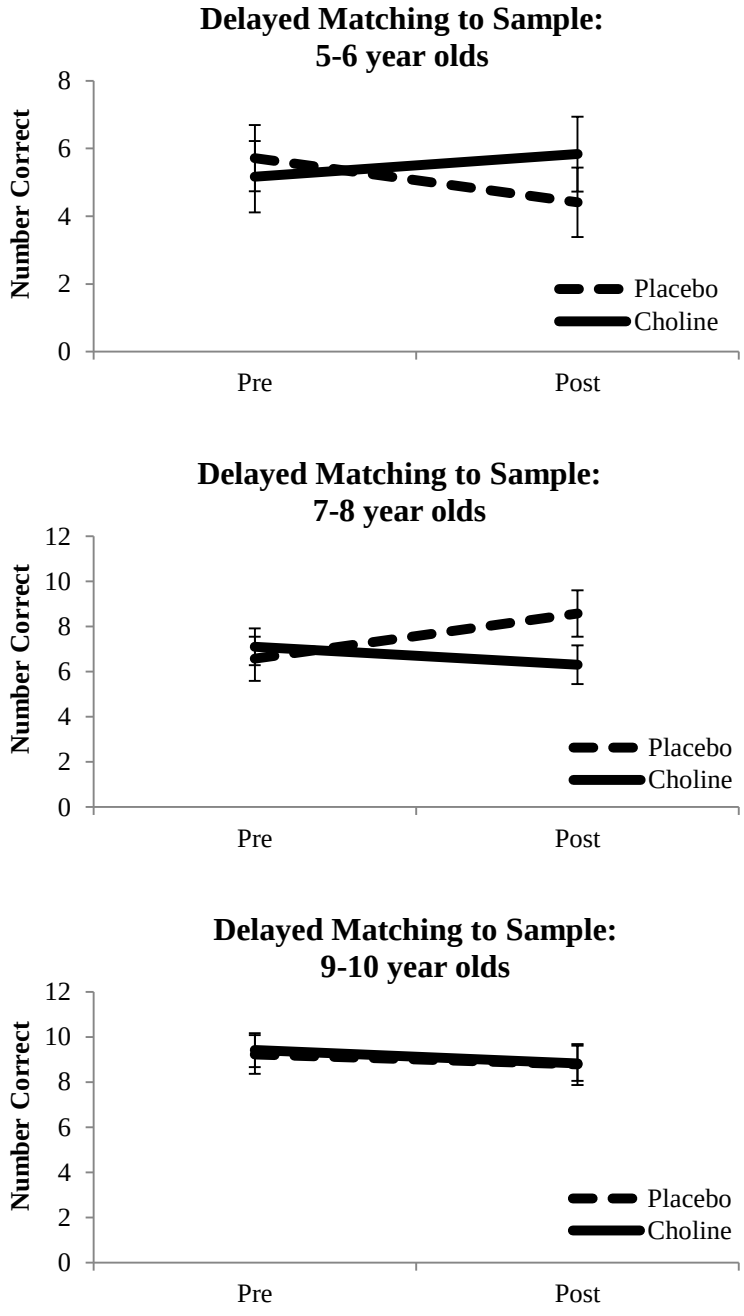


**Figure 8**. Mean nonverbal memory scores from the Delayed Matching to Sample task for the choline and placebo groups within each age group (5-6 years, 7-8 years, 9-10 years).

**The Contribution of Baseline Level of Severity**

Another factor that may differentially affect how children respond to treatment is severity of impairment due to prenatal alcohol exposure. Baseline degree of severity was operationalized two ways: (1) the level of cognitive impairment at baseline (i.e., pre-test scores) and (2) diagnosis of FAS vs. non-FAS. Primary data analyses were repeated, considering these factors in the model, to evaluate the effect of severity on treatment outcomes.

First, (1) bivariate correlations were performed between cognitive performance at pre-test and change scores from pre- to post-test on each outcome measure. Significant correlations were followed up with hierarchical multiple linear regression analyses. Age (if appropriate) and treatment group were entered as covariates on Step 1, baseline score at pre-test was entered on Step 2, and the interaction between Baseline Score x Group on Step 3. The Baseline Score x Group interaction was significant for Latency on the Quotient ($\Delta R^2 = .13$; $\Delta p = .01$) and Grooved Pegboard dominant and nondominant hands ($\Delta R^2$s $> .05$; $\Delta p$s $< .03$). For Latency, subsequent regression analyses within each group revealed that baseline severity was a significant predictor for the choline group ($\beta = -.655$, $p < .001$) and not the placebo group ($\beta = .344$, $p = .06$), such that participants with longer latency scores (i.e., greater inattention) at pre-test showed greater improvement at post-test with choline supplementation. For Grooved Pegboard, follow-up analyses within each group demonstrated that poorer baseline motor performance was significantly related to greater improvement at post-test in the placebo group compared to the choline group ($p$s $< .001$). Finally, (2) when Diagnosis was included in the general linear model

as a between-subjects factor, the Group x Time x Diagnosis interaction was not significant for any measure.

**Predictors of Treatment Outcome**

To guide selection of subsequent hierarchical regression analyses, bivariate correlations between intervention adherence, daily dietary choline intake, and daily total choline intake and cognitive performance at post-test were first performed. Subsequent hierarchical multiple linear regression analyses were performed to assess the unique influence of each predictor on cognitive performance at post-test, after accounting for age, treatment group, and cognitive performance at pre-test. Age (if significantly related to the dependent variable), treatment group, and cognitive performance at pre-test were entered as covariates on Step 1 (Model 1), the predictor variable was entered on Step 2 (Model 2), and the interaction term between predictor variable and Group was entered on Step 3 (Model 3).

**Intervention Adherence**. Treatment compliance, according to returned treatment diaries (i.e., percent of days the treatment was reported to have been taken), demonstrated a statistically significant, positive correlation with PRM number correct at post-test ($r$ = .33, $p$ = .02). Hierarchical multiple linear regression analysis was performed with PRM number correct at post-test as the dependent variable. Model 1 was statistically significant ($R^2$ = .34, $p$ < .001), and model fit was significantly improved with the addition of the moderator variable ($\Delta R^2$ = .07, $\Delta p$ = .03; Model 2); treatment compliance emerged as a statistically significant predictor of PRM number correct, $\beta$ = .265, $p$ = .03, after controlling for age, performance at pre-test, and treatment group. Model fit further

improved with the addition of the interaction term ($\Delta R^2$ = .11; $\Delta p$ = .003; Model 3); both the main effect of treatment compliance, $\beta$ = .607, $p$ < .001, and the Group x Compliance interaction, $\beta$ = -5.75, $p$ = .003, were significant predictors of PRM number correct, after controlling for age, performance at pre-test, and treatment group. Follow-up of the interaction effect revealed that percentage of diary days taken was positively associated with PRM number correct, with increased treatment compliance related to improved memory performance, in the placebo group, $\beta$ = .528, $p$ = .002, but not the choline group, $\beta$ = -.069, $p$ = .71, which was opposite to expectations. The percentage of diary days taken was also significantly negatively correlated with Movements ($r$ = -.42, $p$ = .006) and Displacement ($r$ = -.42, $p$ = .007) on the Quotient ADHD System at post-test. However, subsequent hierarchical regression models did not reveal any statistically significant associations between compliance and hyperactivity after age, performance at pre-test, and treatment group were taken into account ($p$s > .09).

Treatment compliance, according to returned treatment bottles (i.e., percent of liquid consumed), demonstrated a statistically significant, positive correlation with SWM strategy at post-test ($r$ = .34, $p$ = .01). Subsequent hierarchical multiple linear regression analysis revealed that Model 1 was not statistically significant ($R^2$ = .05, $p$ = .31) but Model fit was significantly improved with the addition of the moderator variable ($\Delta R^2$ = .10, $\Delta p$ = .02; Model 2); treatment compliance emerged as a statistically significant predictor of SWM strategy, $\beta$ = .321, $p$ = .02, after controlling for performance at pre-test and treatment group. The direction of this relationship, however, was contrary to expectations, as increased treatment compliance was associated with less efficient

planning. Model fit did not significantly improve with the addition of the interaction term ($\Delta R^2$ = .01; $\Delta p$ = .49; Model 3).

**Dietary Choline Intake**. Average choline dietary intake level demonstrated a statistically significant, positive correlation with DMS number correct ($r$ = .34, $p$ = .02) and negative correlation with Area on the Quotient ($r$ = -.32, $p$ = .04) at post-test. Hierarchical multiple linear regression analyses were performed with DMS number correct and Area at post-test as dependent variables. Of these models, dietary choline intake only emerged as a marginally significant predictor of DMS number correct, $\beta$ = .213, $p$ = .06, after age, performance at pre-test, and treatment group were taken into account ($R^2$ = .50; $\Delta R^2$ = .041, $\Delta p$ = .06; Model 2). Specifically, there was a trend toward a positive association between dietary choline and DMS number correct, with higher dietary choline levels related to improved memory performance at post-test. Model fit did not significantly improve with the addition of the interaction term ($\Delta R^2$ < .001; $\Delta p$ = .92; Model 3), suggesting that those who consumed higher levels of dietary choline were not more responsive to choline treatment than placebo.

**Total Choline Intake**. Average daily total choline intake (i.e., consumed in both diet and intervention) was not significantly correlated with any measure of cognitive performance.

Chapter III, in part, is currently being prepared for submission for publication of the material. Nguyen, T. T.; Risbud, R. D.; Chambers, C.; & Thomas, J. D. The dissertation author was the primary investigator and author of this material.

# IV. DISCUSSION

The present study was a randomized, double-blind, placebo-controlled trial that sought to examine the effectiveness of a 6-week, 625 mg/day choline supplementation intervention for 5-10 year-old children with FASD. The primary aim of the study was to compare cognitive performance between children who received the intervention relative to children who received placebo treatment. It was hypothesized that participants receiving choline supplementation would demonstrate improvements in memory, executive function, and attention/hyperactivity. Secondary aims of this study were to examine the impact of treatment adherence and dietary choline intake on outcomes. Finally, this study provides further information on the feasibility and tolerability of choline supplementation in a child clinical population as well as on the nutritional status of children with heavy prenatal alcohol exposure.

## Feasibility and Tolerability

Analysis of adherence and adverse events data indicated that the study demonstrated high feasibility and tolerability. Ninety-five percent of participants who initiated treatment at the baseline assessment session returned for the follow-up assessment session. Of these participants, 98% returned treatment diaries and bottles, both of which indicated compliance rates of 96% for the sample. These compliance rates are higher than those typically reported in clinical trials of medical regimens, which range from 15-93%, with average rates estimated to be 50% (Kaplan & Simon, 1990; Urquhart, 1991; Wright, 1993), and are comparable to complaince rates from Phase 1 of Dr. Wozniak's choline supplementation trial for children with FASD (Wozniak, et al., 2013)

and other trials of nutritional supplementation in children of similar ages (Bilici, et al., 2004; Richardson & Montgomery, 2005; Richardson & Puri, 2002). The low rate of dropout and high rate of treatment adherence indicated that caregivers were highly motivated to comply with treatment for their children; it might also speak to the helpfulness of weekly phone visits. Future studies would be well served to include a measure at the post-assessment session to assess caregivers' levels of motivation, the helpfulness of the phone visits, and how confident caregivers felt in the effectiveness of the treatment, in order to better understand factors moderating or mediating treatment adherence or nonadherance.

No serious adverse events were reported in this study. Although the number of children who reported at least 1 adverse event in any category was significantly higher for the choline group, treatment groups did not differ on the number of children who reported adverse events in any category, suggesting that the tolerability of the choline and placebo treatments were equivalent. Together, data on compliance and adverse events suggests excellent feasibility of conducting the study and good acceptability of choline supplementation.

During the course of the study, two published studies, which associated a metabolite of choline, trimethylamine-N-oxide (TMAO), with risk for atherosclerotic heart disease (Tang et al., 2013; Wang et al., 2011), were brought to light. The first study (Wang, et al., 2011) reported that atherosclerosis-prone (APOE--) mice that were fed very high levels of choline or TMAO showed greater aortic root atherosclerotic plaque compared to control animals. In a follow-up epidemiological study (Tang, et al., 2013), the authors found an increase in TMAO after consuming phosphatidylcholine and a

relationship between plasma TMAO levels in patients undergoing coronary angiography and cardiovascular events over a 3-year follow-up period. In contrast to these findings, one study in hamsters demonstrated an inverse relationship between plasma TMAO and atherosclerosis (Martin et al., 2009), and another clinical study found that choline improved symptoms related to the chronic cerebrovasculopathy in patients with atherosclerosis (Rossi & Zanardi, 1993). Additionally, an editorial accompanying the Tang et al. (2013) paper in the *New England Journal of Medicine* highlighted the importance of further examining the new model but also cautioned that much remains to be determined in the precise role of TMAO in atherothrombogenesis (Loscalzo, 2013).

Upon learning of these findings, the investigators promptly consulted with a variety of experts, including Dr. Steven Zeisel, an expert on choline and a study consultant, Dr. Howard Taras, the study safety consultant, Dr. Paul Grossfeld, an independent pediatric cardiologist, and the DSMB to discuss the significance of the data and risk to study participants and take precautionary action. From these discussions, it was concluded that, given the available data, a direct causal relationship between choline/TMAO and cardiovascular disease could not be established at this time, and that the relevance of these findings to children ages 5-10 years without risk for heart disease is unclear. Of note, the mean age of the participants in the Tang et al. (2013) study was 63 years, and all subjects had at least single vessel coronary disease. The group also had elevated fasting glucose levels, high rates of diabetes, high rates of hypertension, and elevated rates of previous myocardial infarction. The Institutional Review Board was informed of all findings and proceedings.

**Nutritional Status**

Examination of participants' dietary nutrient intakes revealed that children with prenatal alcohol exposure have poorer nutritional status compared to similar-age, typically developing children in the U.S. Despite similar caloric intake to the NHANES sample, children with FASD consumed fewer vitamins and minerals, suggesting that they may be eating less nutrient dense foods. The Werts study (2014) published earlier this year reported that 25% of children with FASD (ages 3-13 years, $M$ = 9.6 years) had recurrent behavioral problems related to feeding and eating habits, including being picky eaters or having poor appetites, constant snacking, and never seeming full or satisfied, which might influence their consumption of nutritious foods. A caveat, however, should be noted that this study did not include a control group, which limited the ability to make direct comparisons to a typically developing sample and, as such, comparisons were made to age- and sex-matched U.S. norms. Moreover, children with FASD did not meet recommended dietary intakes levels established by the IOM for several important nutrients, including dietary fiber, calcium, potassium, vitamin E, vitamin K, omega-3 fatty acids, and choline. These findings extend results from a previous study, which indicated that preschoolers with FASD had poor nutritional status compared to typically developing children and Dietary Reference Intakes (Fuglestad, et al., 2013), and demonstrate that nutritional inadequacy continues among older, school-aged children. Across these studies, the nutrients consistently found to be in deficit were choline, calcium, potassium, vitamins D, E, and K, and essential fatty acids. As adequate nutrition is critical for healthy brain and cognitive development (Nyaradi, Li, Hickling, Foster, &

Oddy, 2013), nutritional insufficiency may contribute to impaired cognition and behavior in this population, and these data illustrate that this is an area of needed remediation.

**Aims 1–4: Cognitive Performance**

While direct comparisons can be challenging due to differences in participant age ranges and scores utilized across studies, in general, participants' performances on cognitive measures in this study were comparable to those previously found in children with FASD from other studies at the Center for Behavioral Teratology (e.g., Infante et al., 2014). Results from both the ITT and completer samples indicated that participants did not differ in memory performance over time (Aim 1). Across three tasks assessing nonverbal object and visuospatial memory, ITT data did not support the hypothesized differential improvement in memory scores between the choline and placebo treatment groups from baseline to post-assessment (i.e., Group x Time interaction). Although a main effect of Time was observed across groups on the PAL task, this finding likely represents change due to practice effects across multiple test administrations or to brain development and cognitive maturation, although the relatively short time span between pre- and post-assessment suggests the latter is a less likely explanation. Similarly, within completers, results indicated no differential change between groups in memory performance across time. Examination of groups separately indicated that effect sizes were small for both choline and placebo groups, further suggesting that the lack of significant Group x Time interactions was not likely due to insufficient sample size but rather lack of a true treatment effect.

Analysis of executive function measures revealed similar findings (Aim 2). Neither the ITT nor completer sample demonstrated any group differences across the five tasks assessing response inhibition, cognitive fluency, working memory, and planning over time. Although the ITT data revealed significant improvements in inhibitory control, cognitive fluency, and spatial working memory across both groups at post-assessment, there was no evidence of differential changes by group that would support the proposed hypotheses; these findings, again, are more suggestive of practice effects. Results from the completer sample showed no differences across or between groups over time. Effect sizes were small to minimal in both groups without any meaningful differences between groups, indicating a lack of a true treatment effect.

Similarly, for all measures of the Quotient ADHD System, attention performance did not significantly improve for either group over time (Aim 3). Across the measures of hyperactivity, inattention, and impulsivity, ITT and completer data did not support the hypothesized differential improvement in scores across the choline and placebo groups from baseline to post-assessment. Again, effect sizes were very small for both treatments groups, indicating no real change or effect of the treatment on performance.

Finally, we were interested in examining the effect of choline supplementation on motor performance as a negative control (Aim 4). Preclinical studies have demonstrated a specific effect of choline on hippocampal and frontal cholinergic systems but not cerebellar function (Thomas, et al., 2004; Thomas & Tran, 2012). Thus, it was hypothesized that the treatment would demonstrate a dissociative effect, in which behaviors associated with the function of the hippocampus or frontal cortex would be mitigated (i.e., memory, executive function, attention) but not those related to the

cerebellum (i.e., motor coordination), although early analyses from Phase 1 of Dr. Wozniak's choline intervention study found a moderate effect size of choline supplementation on a measure of fine motor functioning (Wozniak, unpublished data). In the ITT sample and among completers, both groups exhibited improved psychomotor coordination over time on the Grooved Pegboard task, and, indeed, there was no differential effect of treatment on motor performance from pre- to post-test. However, given null findings within the other domains, evidence for a dissociation was not supported by the data.

Given the lack of significant findings, additional analyses were performed to explore subgroups of children who may have differentially responded to treatment. First, we investigated whether children in various age groups may have been more or less responsive to choline supplementation. However, the only observed Group x Time x Age Group interaction for DMS revealed contradictory and seemingly spurious effects. Thus, we did not find consistent evidence that choline supplementation benefitted a particular age group of children over another and were unable to establish a therapeutic window for choline within the age range examined. Second, results from our analyses of the relationship between baseline level of cognitive impairment and treatment outcomes were inconsistent and inconclusive. Previous research in normal adults has demonstrated that participants who were cognitively vulnerable or who had lower levels of baseline performance prior to treatment were most amenable to choline supplementation (Ladd, Sommer, LaBerge, & Toscano, 1993; Sitaram, Weingartner, Caine, & Gillin, 1978). Based on these studies, it was hypothesized that choline supplementation would more likely be efficacious among children who were more severely impaired. The only

measure for which this relationship was found was on a variable of response latency on the Quotient. On the other hand, analyses for Grooved Pegboard revealed that children in the placebo group who were more impaired showed greater improvement, which would not be expected. Therefore, these findings appear spurious and may be attributable to regression to the mean (Barnett, van der Pols, & Dobson, 2005; Yudkin & Stratton, 1996). Analyzing effects based on FAS diagnosis was more problematic considering that only approximately half of study participants were able to receive a formal dysmorphology examination to determine FAS diagnosis, and confirmed diagnosis greatly restricted sample size (e.g., only 3 children with FAS in each the placebo and choline groups). Thus, additional research is needed to further explore this hypothesis.

Parent-report data from this study were also consistent with neuropsychological findings and revealed no effect of choline treatment on parent-reported behavioral problems and executive dysfunction, as measured by the Child Behavior Checklist and Behavioral Rating Inventory of Executive Function.

The null findings of this research are surprising given the strong preclinical data suggesting beneficial effects of choline supplementation in animal models of prenatal alcohol exposure. The extant preclinical literature has provided strong evidence for structural and functional changes in brain regions of the hippocampus and cortex following choline supplementation (e.g., Craciunescu, et al., 2003; Glenn, et al., 2007; Williams, et al., 1998), as well as improved neurobehavioral performance on tasks of spatial learning and memory, attention, and executive function—in both typically developing (McCann, et al., 2006) and alcohol-exposed subjects (Ryan, et al., 2008; Thomas, et al., 2009; Thomas, et al., 2007; Thomas, et al., 2004; Thomas, et al., 2010;

Thomas, et al., 2000; Thomas & Tran, 2012). Currently, the results of the present study do not support choline supplementation to be an effective intervention in children with FASD. However, this study is only one of the first to translate animal research to a human clinical trial of choline supplementation in FASD. Given the early stage of this field of research, there are considerable methodological factors to be considered before conclusions can be made. Consequently, a number of factors might account for failure to find potentially beneficial effects of choline.

First, one possibility may be that the children enrolled in this study were too old for the intervention to be successful. Cognitive interventions for neurodevelopmental disorders are dependent on early brain plasticity—the ability of the brain to be modified by external factors (e.g., treatment, experience, behavior, trauma)(Nelson, 1999, 2000). The efficacy of any given intervention depends on the capacity of the brain to compensate or reorganize during a particular window of time (Nelson, 2000), and one of the main challenges of designing and implementing an early intervention program is to determine the critical period when altering neural function is most effective. Although the preclinical data suggest that the choline's critical period is quite large and effects can be seen into later developmental periods (Ryan, et al., 2008; Schneider, et al., 2008; Thomas, et al., 2000), choline supplementation earlier in development has greater benefits (Ryan, et al., 2008; Thomas, et al., 2010). Moreoever, there can be substantial issues in leaping from an animal developmental timeline to that of humans, and human brain development may not always map seamlessly onto a rodent model of brain development. In humans, much of the hippocampal formation develops within the first two years of life with hippocampal differentiation and synaptogenesis occurring until the fifth year (Nelson,

2000; Rice & Barone, 2000; Utsunomiya, Takano, Okazaki, & Mitsudome, 1999). Further, hippocampal-dependent memory has been show to mature until the fourth and fifth years, which suggests that current study participants may have been outside the timeframe of choline's efficacy. Alternatively, the prefrontal cortex is one of the last cortical regions to undergo development, and myelination is continues through adolescence and even late adulthood (Benes, Turtle, Khan, & Farol, 1994; Fuster, 1980; Klingberg, Vaidya, Gabrieli, Moseley, & Hedehus, 1999), leaving open a possibility for interventions to shape executive systems in later childhood and that choline may have more than one therapeutic window throughout development. Data from the current study suggest that choline's therapeutic potential does not fall within the age range of 5-10 years. In contrast, studies of nutritional interventions in populations of children with other developmental disabilities have found supplementation during later periods of childhood (i.e., ranging from ages 5-12) to be effective on cognitive and behavioral outcomes (Harding, et al., 2003; Joshi, et al., 2006; Richardson & Montgomery, 2005; Richardson & Puri, 2002). Thus, further research is still needed to understand the therapeutic window of nutritional interventions.

Related to the above, the treatment duration examined in this study may have been too short. In preclinical studies, the period of choline administration ranged from 10-20 postnatal days, which is equivalent to years in human development. Data from these studies have also suggested that choline's effects are long-lasting due to permanent changes in brain development, rather than an acute effect (Thomas, et al., 2000). Thus, it is possible that children did not receive supplementation long enough to induce long-lasting structural and functional changes in the CNS and, subsequently, behavioral

change. As previously discussed, choline supplementation could potentially alter brain development through several mechanisms, including acute changes in cholinergic activity, metabolic imprinting of cholinergic systems, as well as through cellular membranes, signaling pathways, and epigenetic modification of gene expression. The rationale for investigating a shorter treatment duration was based on extant literature, which supported the effects of acute choline supplementation on cognitive function in other clinical populations. With a shorter period of administration, a possible mechanism of choline's action is through enhancing acute brain acetylcholine levels, which can also facilitate improved learning and memory (Sitaram, et al., 1978), rather than through long-term neurological changes. For example, older adults with memory deficits have shown significantly improved immediate and delayed word, story, and object memory following supplementation with cytidine diphosphocholine (a precursor to choline) for periods lasting 4 weeks (Alvarez et al., 1997) and 3 months (Spiers, Myers, Hochanadel, Lieberman, & Wurtman, 1996). Another double-blinded study found that normal college students who were supplemented with 25 g of phosphatidylcholine (equivalent to approximately 3.25 g of choline) demonstrated improved explicit memory on a serial learning task only 90 minutes following ingestion (Ladd, et al., 1993); interestingly, the results of this study were primarily driven by improvements in participants with slower rates of learning. Similarly, another study found that healthy young adults exhibited improved serial encoding and recall of words within 90 minutes of administration of a single dose of 10 g of choline, and that the degree of enhancement was inversely related to participants' baseline performance on placebo treatment (Sitaram, et al., 1978). These studies suggest that a short-term intervention of choline has the potential to be efficacious,

particularly in subjects who are cognitively vulnerable—as are children with FASD. Nevertheless, while the participants in these studies were cognitively vulnerable, they were still normal volunteers, perhaps indicating that acute cholinergic supplementation may not be sufficient in cognitively impaired populations, i.e., enough to overcome developmental brain damage resulting from prenatal alcohol exposure.

The doses administered in the aforementioned studies of acute choline administration were also quite large (e.g., 10g of choline in the Sitaram, et al., 1978 study, which is approximately 18-25x the adequate intake levels for adults; see Table 1). This raises the possibility that the dose of choline supplemented in the current study were too low (i.e., approximately 1.7-2.5x the adequate intake level), despite early data from Phase 1 of Dr. Wozniak's study, which demonstrated that this level of oral choline supplementation was sufficient to increase serum choline and betaine concentrations (Wozniak, et al., 2013). The prescribed dose in this study was conservatively chosen to be higher than choline levels children typically consume in their daily diets (Fuglestad, et al., 2013), but low enough to be below the tolerable upper intake level, in order to minimize potential adverse effects and increase the tolerability of the intervention and feasibility of completing the study. Thus, an appropriate dose of choline supplementation that may potentially be efficacious remains to be established. Given the relatively low rates of reported adverse events in this study and equivalent number of reports in both treatment groups, future studies might explore higher doses of choline supplementation.

Another important consideration is the form of choline administered, which may potentially facilitate or hinder treatment effects. Cholinergic precursors have been widely administered as potential treatment for patients with neurodegenerative and vascular

dementias, and results of these studies have been mixed, with some showing memory improvements in patients (Levy, 1982; Little, Levy, Chuaqui-Kidd, & Hand, 1985), whereas others have not (Brinkman et al., 1982; Fitten et al., 1990; Higgins & Flicker, 2000; Weinstein, Teunisse, & van Gool, 1991). Further evaluation of these studies has raised the question of whether choline is truly ineffective in this population or if just inefficient forms are being used (Parnetti, Mignini, Tomassoni, Traini, & Amenta, 2007). For instance, many of the studies reporting negative results used choline compounds in the form of choline salts (e.g., choline chloride) and lecithin (i.e., phosphatidylcholine), which are not ideal for enhancing brain levels of acetylcholine (Amenta, Parnetti, Gallai, & Wallin, 2001). On the other hand, choline alphoscerate (otherwise known as glycerophosphocholine, the form of choline administered in this study) was better at augmenting acetylcholine availability and release in the brain (Sigala et al., 1992), and was found to improve clinical status in dementia disorders of degenerative, vascular, and combined origins (Parnetti, Amenta, & Gallai, 2001). Examples from the dementia literature support the form of choline administered the present study, although various other factors may interact with this choline form, including clinical population, age, treatment duration, and dose.

Furthermore, it is possible that choline may effect change in cognitive and behavioral domains that were not measured in this study, or the instruments used in this study may not have been sufficiently sensitive to detect improvements. The neuropsychological measures administered in this study were carefully selected to be as analogous as possible to those tested in preclinical models, as well as for their sensitivity to intervention and nutritional supplementation (Anderson, Polcari, Lowen, Renshaw, &

Teicher, 2002; Bryan, Calvaresi, & Hughes, 2002; Klingberg et al., 2005; Miranda, Presentacion, & Soriano, 2002; Mwanri, Worsley, Ryan, & Masika, 2000; Rhodes, Coghill, & Matthews, 2004; Teicher, Lowen, Polcari, Foley, & McGreenery, 2004; Teicher, Polcari, & McGreenery, 2008; van den Briel et al., 2000). However, inherent differences exist between animal behavioral and human cognitive testing, which may have impacted the ability to detect treatment effects. Further research is needed to examine instruments that might be most sensitive to change associated with choline supplementation, including more comprehensive assessment of global cognitive function in addition to focused assessment of hippocampal and prefrontal functions.

It is not uncommon that therapeutic efficacy in animals does not translate to humans in clinical trials (Hackam, 2007; Hackam & Redelmeier, 2006). Although the rodent model is the most widely used and validated in the study of FASD, especially for behavioral studies (Cudd, 2005), animal models cannot adequately mirror all aspects of human pathophysiology. Experimental animals rarely have mood and psychiatric comorbidities and are not vulnerable to the range of competing and interacting social and environmental demands and interventions as those experienced by children with FASD (Hackam, 2007). Sixty-four percent of children in this study were reported to have at least one comorbid psychiatric diagnosis based on caregiver interview and questionnaires. In children more so than animals, especially in a clinical population of children with high rates of comorbid psychopathology (Fryer, et al., 2007), cognition, behavior, and mood are closely intertwined; fluctuations in behavior and psychiatric status may increase demands on children's attentional system, alter their ability to emotionally self-monitor, and, consequently, influence cognitive performance apart from their ability dependent on

brain function, alone. Thus, targeting change in cognitive status is likely more complex in humans than in animal models, as cognitive performance likely interacts with and may be masked by other factors, including exacerbations in behavioral presentation. These are practical challenges of conducting a clinical trial that cannot be completely captured or accounted for in experimental animal trials.

Additionally, individuals can vary widely in their dietary requirements for choline, and common genetic variations likely underlie this difference (da Costa et al., 2006; Zeisel, 2007). As the metabolism of choline, methionine, and folate are highly interrelated, several metabolic pathways influence how much choline is required from the diet, including the dietary availability of other methyl donors (e.g., folate) and endogenous de novo biosynthesis of choline (Zeisel & Blusztajn, 1994). In each of these pathways, single nucleotide polymorphisms (SNP) in specific genes can influence metabolic efficiency and, consequently, the dependency of the body for dietary choline (Zeisel, 2007, 2011a, 2011b). Consequently, it is possible that genetic polymorphisms may interact with choline supplementation, such that children with SNP-induced metabolic inefficiencies may be less responsive to intervention, while those without these genetic variations may be more likely to benefit from supplementation. As part of this clinical trial, saliva samples were collected to obtain DNA samples for genetic analysis. Samples will undergo genome-wide profiling of relevant polymorphisms related to these metabolic pathways that may underlie inter-individual variability in susceptibility to choline supplementation. These SNPs may identify genetically different subpopulations of alcohol-exposed children who may require higher levels of supplementation to overcome metabolic inefficiencies or, alternatively, those who may be more readily

responsive to (or require lower levels of) supplementation. Understanding these differences may help us target and customize interventions at the level of the individual, rather than solely at the group level. Furthermore, choline intervention and presence of SNPs in these choline metabolic pathways may also be moderated by dietary choline intake, such that children with SNPs, who also consume a low-choline diet, may be at greater risk for impairment (Zeisel, 2011b) and/or may require an even higher level of supplementation. Thus, with the addition of genotyping data, future analyses will include a model that considers both SNPs and dietary choline intake as potential moderators or mediators of treatment outcomes.

Along the same lines, it is challenging to control dietary intake and general nutrition in clinical studies compared to animal models. While the analyses attempted to examine and account for dietary choline as a moderator of treatment outcome, dietary choline availability and overall nutritional status may have still confounded treatment results. The fact that dietary choline was related to memory performance, but yet choline supplementation was not found to improve cognition, suggests that perhaps dietary choline is a proxy for overall nutritional status or related to dietary levels of another nutrient that may be influencing cognition. Existing literature on the effects of diet and supplementation on behavior and cognition in children suggests that micronutrient supplementation can improve intelligence and cognition in children who are malnourished or who have inadequate dietary status but not in their adequately nourished peers (Bellisle, 2004). Future research should focus on developing better measures of overall diet quality and understanding children's long-lasting nutritional status, which

might help us better understand the nature of nutrition and supplementation in this population.

While the current results do not support the hypotheses, they raise other important questions about choline supplementation and the role of nutritional interventions, in general. One interpretation of the current data is that choline supplementation, alone, is not sufficient to produce meaningful changes in cognition. It may interact synergistically with other nutritional or pharmacological interventions, which together may have a more impactful influence on neurocognitive development. This hypothesis is supported by early data from the Ukraine prenatal nutritional supplementation study (Keen, et al., 2010), showing that multi-micronutrient supplementation improves mental development in infants prenatally exposed to alcohol (Coles, et al., 2011) and that the addition of choline leads to improved neurophysiological encoding and memory independent of micronutrient supplementation alone, although the effects of choline alone were not evaluated (Kable, et al., 2012; Kable, et al., 2011). Another closely related study examined the effect of choline alphoscerate (400 mg/day, equivalent to 162 mg/day choline) in combination with neuroleptic maintenance therapy among children with mild-to-moderate autism (Krasnoperova, Simashkova, & Bashina, 2004). After only 8 weeks, positive therapeutic effects were observed in 89% of children in areas of general behavior, development of social and communicative skills, speech disturbances, motor development, and learning activity/productivity.

Finally, augmenting overall nutrition through food and multi-nutrient supplementation may be more beneficial than any single nutrient intervention (Barrett & Radke-Yarrow, 1985; Waber et al., 1981), especially as deficiencies in macronutrients

and micronutrients during pregnancy may detrimentally affect neurodevelopment, and children with FASD are at especially high risk of undernutrition. Although some single micronutrient interventions have been shown to benefit children's mental development in clinical studies (Bryan et al., 2004), several meta-analyses and systematic reviews suggest that multi-micronutrient interventions have advantages over single nutrient supplementation in improving cognitive development and other outcomes in children (Benton, 2001; Eilander et al., 2010; Ramakrishnan, Nguyen, & Martorell, 2009).

**Predictors of Treatment Outcome**

Results from the secondary aim revealed that dietary choline intake did not moderate treatment outcomes. Although we did not support the proposed hypothesis that children with lower levels of dietary choline would be more likely to improve with choline treatment (i.e., Group x Dietary Choline interaction), results indicated that dietary choline intake was a marginally significant predictor of memory performance at post-test, across both groups. Although not statistically significant, findings from this analysis indicated that there was a trend towards a positive relationship between dietary choline levels and memory performance. Although recent data have shown that children with FASD have disordered eating problems and are nutrient deficient compared to their typically developing peers and IOM Dietary Reference Intakes (Fuglestad, et al., 2013; Werts, et al., 2014), no previous study to my knowledge has reported on the relationship between children's dietary status and the functional implications on their cognition and behavior. Although the lack of statistical significance precludes firm conclusions, this

result highlights an area of further research that is needed to understand the nature of nutritional status in FASD and its role in neurocognition.

Similarly, treatment compliance was not predictive of improved cognitive performance at post-test. For percentage of diary days taken, although the addition of the Group x Compliance interaction term on Step 3 of the linear regression analyses resulted in a significant increase in explained variance in object recognition memory at post-test, the direction of the relationship was contrary to expectations, with increased treatment compliance related to improved memory performance in the placebo group but not the choline group. Compliance would not be expected to have a relationship to treatment outcomes in the placebo group, as these children did not receive the active intervention. An alternative explanation for this finding might be that compliance was related to a third unmeasured confounding factor that may have influenced memory performance at post-test. For example, parental adherence behavior may have been concomitant with additional care taking or expectations that contributed to better outcomes (DiMatteo, Giordani, Lepper, & Croghan, 2002). Treatment compliance (i.e., diary days taken or liquid consumed) did not exhibit a differential association with any other cognitive variables in the choline group, after covariates were taken into account. These results are not inconsistent with research available on the adherence-outcome relationship. The relationship between adherence and treatment outcome is complex and can be moderated by many factors, such as genetic variations in response rates, efficacy of treatments, SES, and parent psychopathology and stress (DiMatteo, et al., 2002; Nock & Ferriter, 2005).

**Strengths and Limitations**

Several limitations to the present study should be acknowledged. First, although this project exceeded its proposed recruitment goal of $n = 20$ subjects per group, the study may have been limited by issues of power. Early analyses from Phase 1 of Dr. Wozniak's 9-month choline intervention revealed medium effect sizes (Cohen's *d* ranging from 0.29 to 0.59) on neurobehavioral outcomes averaged across 6 and 9 months. The largest effect size was observed on fine motor functioning ($d = 0.59$), followed by short delay memory ($d = 0.42$), and global cognitive functioning ($d = 0.29$) (Wozniak et al., unpublished data). Based on these findings, we estimated medium effect sizes for the dependent variables ($d = 0.59$). Given this estimate, a sample size of $n = 37$ per group would have been necessary to have .80 Power to detect a significant effect. However, the sizes of the non-significant differences were quite small (e.g., $d = .10$), indicating that nearly 2500 subjects would be required to result in statistically significant differences. As such, these likely reflect real non-differences rather than insufficient power.

A broad age range was targeted for inclusion in the current study and performance on almost all neuropsychological measures differed as a function of age. In general, the data show that older children performed better on most neuropsychological measures. Thus, the varying capabilities of the subjects due to differences in level of cognitive development may have diluted potential treatment effects. Sub-analyses were performed with children ages 5-8 years and 9-10 years, and age group was examined as an independent factor to determine if response to treatment varied depending on age. Nonetheless, no effect of treatment was observed in the choline group, although smaller sample sizes within each age group may have precluded the detection of significant

effects. Future investigations might consider targeting a narrower age range to gain a better understanding of choline's effect at specific development periods.

In order to increase the ecological validity of this study, children with comorbid behavioral and psychiatric disorders, such as ADHD, learning disability, oppositional defiant disorder, and conduct disorder, were not excluded from the study, as these diagnoses are common within this clinical population. However, as psychiatric comorbidity has been shown to be risk factor associated with poorer response to intervention (Shea, Widiger, & Klein, 1992) and can be associated with cognitive impairment, independent of alcohol exposure status (Castellanos, Sonuga-Barke, Milham, & Tannock, 2006; Clark, Prior, & Kinsella, 2002; Clark, Prior, & Kinsella, 2000), concomitant psychopathology in the sample may have confounded the results. Moreover, while comorbid psychopathology was assessed using caregiver report, there are inherent limitations in the reliability of informant-reports (Achenbach, McConaughy, & Howell, 1987). Future studies would be well served to include standardized diagnostic assessment measures to assess for current and past psychopathology and explore psychiatric comorbidities as a potential treatment moderator.

Another limitation of this study, as with many clinical trials, is the reliance on indirect methods for measuring participant adherence to the treatment regimen. Participant-kept diaries and pill counting (or in the case of this study, liquid measurements) are some of the most common methods to determine regimen adherence. While these methods provide valuable data, their accuracy can vary widely and can often overestimate actual adherence behavior (Cramer, Mattson, Prevey, Scheyer, & Ouellette, 1989; Matsui et al., 1994). Factors that may influence measurement of true adherence

include forgetting to or deliberately not returning diaries and bottles, accidentally spilling liquid, and purposefully disposing of liquid or falsification of the treatment diary to hide errant administration. While direct methods (e.g., measuring serum choline levels in plasma) may be more valid indicators of whether the treatment was consumed, it is often difficult to assess compliance quantitatively unless serial blood samples are drawn (Farmer, 1999). As such, no measure of compliance is without disadvantages, and it is believed that the methods chosen were the most appropriate given the resources and goals of the dissertation project.

For safety reasons, children were allowed to continue taking any medication concurrently prescribed by a physician, and medication changes during the treatment period did not preclude study participation. As a result, changes in medications or doses may have impacted results upon follow-up testing. Eleven children had reported medication changes during the treatment period, with a significantly greater proportion in the choline group (8% in placebo group, 31% in choline group; $p = .03$). Although caregivers were asked to refrain from administering any cognitive enhancing medications before cognitive testing, it is possible that medications may have influenced testing depending on their washout period. Conversely, a sudden medication washout may have inadvertently exacerbated children's behavioral status, leading to increased excitability, irritability, and/or distraction that may have affected performance, as well.

Relatedly, given that caregivers were informed that the study was trial of choline supplementation on neurocognitive outcomes, it is possible that they may have attempted to increase their children's choline intake via diet or supplementation. However, the 24-hour dietary recall data revealed no significant change in dietary choline or other nutrient

intake over time for either group. Regarding supplementation, parents were explicitly instructed not to change children's nutritional supplements or begin any specific choline supplements during the duration of the study. Although it is possible that caregivers may have done so despite instructions, there was no evidence that this occurred, as all caregivers denied any changes in supplements at the follow-up appointment. Moreover, the likelihood that this may have preferentially occurred in either treatment group was low as the study was double-blinded.

Finally, given resource limitations of the current study, no measure of IQ or global cognitive function was administered. As a result, this precluded the ability to investigate IQ as a potential moderator of treatment outcome, especially as overall cognitive function may be a better predictor of how severely affected an alcohol-exposed child may be.

Despite these limitations, the current study has many notable strengths and adds to the available literature in several respects. A primary strength of this study is its methodological rigor as a randomized, double-blind clinical trial. Randomization serves to balance unknown and/or unmeasurable factors that may bias study outcomes. Within developmental models, these factors include intra-individual characteristics of children or their parents (e.g., parenting style, motivation of parents or children to participate in the intervention) (Duncan, Magnuson, & Ludwig, 2004). As a result of random assignment, treatment groups in this study were equivalent, not statistically differing on any demographic or cognitive performance variable at baseline (see **Tables 3 and 4**). The sample of subjects in this study, collected from two centers across the United States, encompassed a wide range of SES and is representative of the general FASD population.

Another strength was the low rate of participant dropout and adverse events as well as high rate of treatment adherence. This study demonstrates that choline supplemention is feasible and tolerable in children with FASD. Furthermore, this study adds to and extends the current literature focused on understanding the nutritional status of children with histories of prenatal alcohol exposure. Although we did not see improvement in cognitive function with choline supplementation, these data still suggest that there are opportunities to intervene as children with FASD are not meeting important nutritional standards that may affect overall health and growth as well as behavioral and cognitive functioning.

Although preclinical studies have demonstrated that choline supplementation can ameloriate abnormal neurodevelopment and behavioral deficits associated with prenatal alcohol exposure, this study is one of a very limited set of investigations that have started to translate these data to a clinical population of children with FASD. It is the first known study to examine choline supplementation in children of school-age, around the time during which FASD is most commonly diagnosed. As with the development of any new intervention or therapy for any population, there are immense challenges, and this project designed and implemented the most feasible nutritional intervention program possible given the nascent stage of this field of research as well as the variety of methodological factors that have yet to be understood about choline's effects in children with FASD.

**Summary and Future Directions**

In summary, this project is one of the first investigations to explore whether choline supplementation can ameliorate neurobehavioral deficits in children with FASD. Unfortunately, we did not find evidence that choline, administered at a dose of 625

mg/day for 6 weeks, improves neuropsychological function in the domains of memory, executive function, or attention/hyperactivity. However, these findings, alone, do not close the door on the possibility that choline supplementation, or other nutritional interventions, may improve cognitive function in children with FASD. Considering the challenges associated with translating preclinical research to human clinical trials and the inaugural phase of this field of research, additional studies are still needed to further elucidate and understand possible therapeutic developmental windows of choline, the possibilities of and mechanisms associated with a short- versus long-term treatment duration, and how choline compound and dose level affects outcome. Further studies are also needed to understand the contribution of genetic variations that may play a role in intra-individual responses to choline and nutritional interventions, in general. Nevertheless, although no support for the primary hypothesis was found, the study highlights the feasibility and tolerability of choline supplementation in children with FASD, the inadequate nutritional state of these children, and a trend towards improved memory performance with increased dietary choline. Altogether, this study emphasizes the need for a continued understanding of the role of nutritional status and supplementation in FASD and its contributions to neurocognition, and this project is only the first of, hopefully, many to further examine these relationships.

Chapter IV, in part, is currently being prepared for submission for publication of the material. Nguyen, T. T.; Risbud, R. D.; Chambers, C.; & Thomas, J. D. The dissertation author was the primary investigator and author of this material.

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