A placebo-controlled pilot exploration of cholesterol supplementation for autistic symptoms in children with low cholesterol

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Informed consent: Written informed consent and assent (if the child was capable) was obtained from parents. The authors affirm that human research participants provided informed consent for the use and share of their de-identified information for future research and publication.

ABSTRACT

Objective: This pilot placebo-controlled trial (NCT00965068, 07/28/2009) explored effects of cholesterol supplementation on autism spectrum disorder (ASD) symptoms in children with abnormally low cholesterol. **Method:** Fifteen children age 4-12 with ASD and cholesterol <5th percentile for age/sex were randomized to 225 mg cholesterol vs. matched placebo twice daily for 12 weeks, followed by 12-week open extension. The cholesterol daily dose approximated the amount in two eggs. Measures included the Ohio Autism Clinical Impression Scale (OACIS, primary outcome), and Peabody Picture Vocabulary Test (PPVT-4). Effect sizes were emphasized over statistical tests. **Results:** On the OACIS-Improvement score for overall ASD symptoms, both groups improved, with cholesterol showing nominally (nonsignificantly) more improvement. The cholesterol group improved more than placebo by medium to large effects in OACIS-I nonverbal communication (d=-0.643); and OACIS-I problems with anxiety and fear (d=-0.671). Moreover, cholesterol showed significantly more improvement than placebo in PPVT-4 receptive language (p=0.040). During the 12-week open extension, children originally assigned placebo improved significantly more in OACIS-I social interaction (p=0.026, d=1.170) from end of randomized phase than those receiving cholesterol throughout and improved more by medium to large effects in OACIS-I aberrant behavior (d=0.924), and anxiety/fear (d=0.925). Graphs suggest continued open-trial improvement for the cholesterol group. Adverse events were similar between treatments. **Conclusion:** Cholesterol supplementation of children with ASD and cholesterol below the 5th percentile appears safe. The effect on communication, social interaction, and anxiety/fear looks encouraging; deserving further study, possibly over a longer period or at higher doses.

Keywords: cholesterol supplementation; autism; low cholesterol; Smith-Lemli-Optiz syndrome; complementary/alternative treatment

INTRODUCTION

Background and Significance

Official prevalence of autism spectrum disorder (ASD) has gone from 0.1% to over 1% from 1970 to 2018 (Christensen et al. 2018). Often presenting early in childhood, ASD persists in attenuated form throughout life. Common characteristics include deficits in language development, social communication, and social skills, in addition to restricted/repetitive interests or stereotyped movements and sensory abnormalities (DSM-5, American Psychiatric Association [APA] 2013). The pool of patients included under the diagnosis of ASD has changed in recent years. According to DSM-IV (American Psychiatric Association [APA] 1994), autistic disorder was distinct from Asperger's disorder, Childhood Disintegrative Disorder, Rett's Disorder, and Pervasive Developmental Disorder not otherwise specified (PDD-NOS), but DSM-5 includes autistic disorder, Asperger's, and PDD-NOS in the ASD diagnosis (DSM-5, American Psychiatric Association [APA] 2013). ASD has been coupled with multiple other genetic disorders, including Fragile X syndrome, Angelman Syndrome, Rett syndrome, and Smith-Lemli-Opitz syndrome (SLOS).

Most children with SLOS have ASD (Bukelis, Porter, Zimmerman, and Tierney 2007; Sikora et al. 2006; Tierney et al. 2001). SLOS is an autosomal recessive metabolic disorder caused by mutation in the DHCR7 gene resulting in a deficiency of 7-dehydrocholesterol (7DHC) reductase, an important enzyme in cholesterol anabolism. Patients with SLOS have increased levels of 7DHC and decreased levels of cholesterol (Porter and Herman 2010; Irons et al. 1993). Although the DHCR7 mutation has a carrier frequency of 1.25%, the estimated incidence of SLOS is only 1 in 39,000 births due to most homozygous fetuses not surviving till birth, (Cross et al. 2014). Three-fourths of SLOS participants have pervasive developmental disorder (Sikora et al.

2006), roughly equivalent to ASD. Thurm et al. (2016) found that the low level of functioning observed in some individuals with SLOS may artificially inflate scores on standard autism assessments and that 7DHC and 8 dehydrocholesterol (8DHC) are important biomarkers of the level of functioning.

Although no blinded randomized clinical trials (RCTs) of cholesterol supplementation in SLOS have been published, case reports suggested that cholesterol supplements decreased ASD symptoms (Elias et al. 1997; Nwokoro and Mulvihill 1997; Pauli et al. 1997; Elias and Irons 1995). Additionally, treatment with cholesterol was reported to decrease irritability (Elias and Irons 1995; Nwokoro and Mulvihill 1997), lead to a happier affect (Irons et al. 1993; Nwokoro and Mulvihill 1997; Pauli et al. 1997; Opitz 1999), decrease hyperactivity, improve attention (Elias and Irons 1995), and decrease self-injury (Irons et al. 1993; Ryan et al. 1998). Other problem behaviors that decreased with cholesterol supplementation include aggression (Nwokoro and Mulvihill 1997; Ryan et al. 1998), temper outbursts, trichotillomania, and tactile defensiveness (Nwokoro and Mulvihill 1997). Individuals with SLOS have also been reported to be calmer (Pauli et al. 1997) and more sociable and even began to initiate hugs after cholesterol supplementation (Nwokoro and Mulvihill 1997; Pauli et al. 1997).

Additional improvements noted after cholesterol supplementation were increases in alertness and activity in participants who were abnormally passive (Irons et al. 1993; Ryan et al. 1998), and improved sleep patterns (Ryan et al. 1998). The hearing of two participants was reported improved (Irons et al. 1993) and participants showed an increase in expressive language (Pauli et al. 1997; Opitz 1999). Reports of violent episodes and self-injury also decreased with cholesterol supplementation (Tierney et al. 2001). Based on such reports, cholesterol has become a standard therapy for SLOS (Segatto, Di Giovanni, Marino, and Pallottini 2013). The

improvement of autistic symptoms with cholesterol supplementation led to a hypothesis about the importance of cholesterol in etiology of ASD.

Cholesterol, a precursor to steroid hormones, plays a role in brain function and development. According to Segatto et al. (2013), the brain has about 25% of all cholesterol in the human body. Evidence (Lee and Tierney 2011; Segatto et al. 2013) has shown that cholesterol is a structural component of myelin sheaths and lipid membranes, and it helps to facilitate hedgehog signaling (signaling for proper cell differentiation in embryonic cells). Levels of lipid rafts, which enhance synaptic plasticity, rose with introduction of statins, a cholesterol inhibitor, as a treatment for Fragile X syndrome and Neurofibromatosis Type 1 in animal models (Wang 2014). Modifications of cholesterol synthesis were shown to decrease levels of oligodendrocytes and myelin in rat models with ASD-like symptoms (Cartocci et al. 2018). It has been hypothesized that mechanisms for ASD development may be related to a cholesterol deficit because cholesterol is 1) necessary for normal embryonic and fetal development, 2) required for myelin membranes, 3) a precursor for neuroactive steroids, 4) a modulator of oxytocin receptor function, 5) a component of lipid rafts (domains of the plasma membrane storing high concentrations of cholesterol), 6) a modulator of ligand binding activity in G protein coupling of the serotonin 1A receptor (Aneja 2008); and 7) a precursor for vitamin D and the sex hormones (Aneja 2008; Gillberg et al. 2017).

Although the specific mechanism has not been determined, cholesterol has been shown to play a role in ASD. For instance, lower levels of neurosteroids, which control neurotransmitters and their receptors, were found in ASD patients (Korade, Folkes and Harrison 2013; Frye and Llaneza 2010; Moy et al. 2009; Tierney et al. 2006; Waage-Baudet et al. 2003). The amount of cholesterol entering the brain is regulated by the blood-brain barrier; additional synthesis of

cholesterol takes place in the brain (Segatto et al. 2013; Cartocci et al. 2018). With this in mind, we determined that a study of cholesterol supplementation in children with ASD was warranted.

Aims and Hypotheses

In this pilot study, we aimed to explore the effects of cholesterol supplementation on ASD symptomatology compared to placebo. We hypothesized that (1) within the first 12 weeks (pilot randomized controlled trial), participants in the cholesterol group would improve more than participants in the placebo group in ASD symptomology and (2) within the second 12 weeks (open extension), those originally assigned placebo would also show improvement in ASD symptomology similar in magnitude to what the original cholesterol group showed initially. In addition to our primary analyses, exploratory analyses were conducted to assess whether cholesterol supplementation exerted any alleviating and/or detrimental effects on specific ASD symptoms across a variety of domains, such as communication and other social skills.

Although cholesterol does not efficiently cross the blood-brain barrier, we expected cholesterol to exert effects through the adrenal glands, with cholesterol used to synthesize neuroactive steroids and steroid hormones (Borkowski, Levin et al. 1967, Bochem, Holleboom et al. 2013). The neuroactive steroids promote synaptogenesis and dendrite growth and modulate neurotransmitter receptor activity. Decreased neuroactive steroid levels have been reported in ASD (Strous, Golubchik et al. 2005). Other sterol related abnormalities implicated in ASD include abnormalities in cortisol, and levels of vitamin D, estrogen, progesterone and testosterone (Aneja and Tierney 2008, Lee and Tierney 2011, Gillberg, Fernell et al. 2017).

METHODS

Study Design

The research was approved by The Ohio State University's Institutional Review Board (IRB), and written informed consent and assent (if the child was capable) was obtained from parents. Children ages 4 to 12 with ASD were randomly assigned in a parallel groups design to either 225 mg cholesterol (N = 8) or matched placebo (N = 7) twice daily. The 12-week double-blind randomized controlled trial of cholesterol supplementation (*RCT*) was followed by a 12-week open-label extension (*open extension*). During the open extension, all participants received cholesterol with the same dose schedule as the first 12 weeks.

Supplement

The cholesterol used microencapsulation and contained vitamins C and E. The placebo was composed of methylcellulose with vitamins C and E. A description of the supplement as well as a detailed list with the dose of each ingredient can be found in the supplementary material. We added a multivitamin/mineral formula to the placebo and the cholesterol supplement, to standardize basic background micronutrition for both treatment groups. Although vitamins/minerals themselves can have therapeutic effects (Adams 2015; Adams and Holloway 2015), any improvement in the cholesterol group relative to the placebo group would be due to added cholesterol. The cholesterol and placebo (both in powder form in packets) were administered twice a day. The daily dose of 450 mg cholesterol is approximately equal to the amount of cholesterol in two old-fashioned egg yolks (not the low-cholesterol eggs). The cholesterol dose of 450 mg was chosen based on the lowest dose used to treat individuals with SLOS (Elias et al. 1997; Irons and Elias 1997; Tierney et al, 2010). If children missed a scheduled dose, they were asked to take the missed dose along with the next scheduled dose ("double up").

Parents of participants had to agree to use only supplements supplied by the research study throughout the length of the study. Parents were discouraged from modifying diets and/or active supplementation (i.e., using high cholesterol foods such as egg yolks and cream) in addition to the prescribed dietary supplementation. Adherence was assessed at each clinical visit.

Study Participants

Clinical providers recruited potential participants at The Ohio State University (OSU) and Nationwide Children's Hospital. In addition, resources and advertisements were posted on websites to recruit potential participants. Potential participants were pre-screened for eligibility via telephone prior to coming to the research site for a screening visit.

Inclusion Criteria. Participants were children ages 4 to 12 with rigorously diagnosed ASD and cholesterol at or below the 5th percentile for age and sex, using the National Health and Nutrition Examination Survey (NHANES, 2006) norms. The minimum age of 4 years ensured that most participants maintained a nonverbal mental age of 18 months or greater to support use of the Autism Diagnostic Interview-Revised (ADI-R). The age maximum of 12 years was chosen to lessen the chance of having individuals with mood disorder and irritability that may begin during adolescence (which occurs at a high rate in both ASD and SLOS) and because the individuals with ASD+Hypocholesterolemia may also have abnormal hormonal levels, as occurs in SLOS. We included a broad age range because the blood cholesterol levels between the ages of 4 and 12 years may be more stable if the pattern of variation in blood levels with age is similar to that seen with SLOS. All participants had to meet the Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV, American Psychiatric Association [APA] 1994) diagnostic criteria for Autistic Disorder, Asperger's Disorder, or Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS) by master's-level clinicians who were research-reliable in administering the Autism

Diagnostic Interview-Revised or Autism Diagnostic Observation Schedule. Diagnoses were confirmed by doctoral-clinician review.

Exclusion Criteria. Participants were excluded from the study for a diagnosis of SLOS or known cholesterol synthesis/regulation disorder; an abnormal karyotype; a DSM-IV diagnosis of Rett's Disorder, Childhood Disintegrative Disorder, Schizophrenia, any other psychotic disorder, or substance abuse; a significant medical condition (like heart disease, hypertension, liver or renal failure, pulmonary disease, diabetes, or unstable seizure disorder); changes in anticonvulsant dose in the past three months or a seizure in the past eight weeks; neuroleptic medication; statin medication; dietary cholesterol supplementation in the past three months; or any other medications that affect cholesterol or other lipid levels. All participants had blood samples tested in the CLIA certified Kennedy Krieger Institute Biochemical Genetics Laboratory where cholesterol and the sterol precursors/enzymes 7-dehydrocholesterol reductase, lathosterol and desmosterol were measured. No participant had a biochemical pattern consistent with either SLOS, Lathosterolosis or Desmosterolosis. All participants who were found to have a total cholesterol level in the hypocholesterolemia range had a vial of plasma sent to a laboratory for biochemical testing for SLOS. The biochemical testing involved measuring the ratio of 7-dehydrocholesterol (7DHC) to total cholesterol and represents the standard clinical procedure for detecting SLOS.

Measures

Diagnostic/Eligibility Measures

Stanford-Binet Intelligence Scale: Fifth Edition (SB5, Roid 2003). The SB5 is a battery of cognitive tests used to assess cognitive strengths and weaknesses. Raw scores are converted

into three types of standard scores. The SB5 was used to ensure the inclusion criteria were met for a mental age of 18 months for children with expressive language. Young children were given the nonverbal portion of the SB5 only. Research demonstrated that the SB5 is valid and reliable (e.g., Laurent, Swerdlik, & Ryburn, 1992). It was administered by a master's level psychologist supervised by a post-doc.

Mullen Scales of Early Learning (MSEL) (Mullen 1995). The MSEL are used to assess cognitive functioning in young children from birth to age 5 years and 6 months. The five brief scales measure early cognitive and motor development (i.e., Gross Motor, Visual Reception, Fine Motor, Receptive Language, and Expressive Language). The Early Learning Composite score and the age equivalence scores are derived from the subscale raw scores). For children with impaired verbal skills who did not meet the age criterion to calculate an Early Learning Composite score on the MSEL (i.e., older than 5 years and 6 months), age equivalence scores were used to assess cognitive functioning and to derive an IQ score. The MSEL has been demonstrated to be a valid and reliable instrument, particularly for children with ASD (e.g., Akshoomoff, 2006). It was administered by a master's level psychologist supervised by a post-doc.

Outcome Measures

Clinician-rated Semi-Structured Interviews and Caregiver Report

Ohio Autism Clinical Impressions Scale – Severity and Improvement (OACIS, Butter and Mulick 2006). The OACIS-S is used to measure severity of autistic behavior, while the OACIS-I is used to measure the change in autistic behavior during the trial (Singh et al., 2014). The OACIS-S includes a 7-point Likert scale of (1) Normal, not at all ill, (2) Borderline mentally

ill, (3) Mildly ill, (4) Moderately ill, (5) Markedly ill, (6) Severely ill, (7) Among the most extremely ill patients. The OACIS-I change from baseline in ASD symptomology was rated from 1 (very much improved) to 8 (very much worse) with 5 (no change). By definition, all participants have a baseline rating of 5 (no change from baseline). Note that the usual scoring on the OACIS-I is 1 (very much improved) to 7 (very much worse). We modified this to a 1 to 8 scale with 5 representing no change to obtain a finer-grained rating of improvement. General and specific ASD symptomology were rated on the same scale and are listed in Table 2. The OACIS-S was administered by a post-doc at weeks 0, 12, and 24. The OACIS-I was administered by a post-doc at weeks 2, 6, 12, and 24. The overall symptom rating was the primary outcome, with the nine specific symptoms as the main secondary outcomes. The OACIS is an observer-rated scale and has been used on several ASD clinical research trials (e.g., Arnold et al., 2012; Frye et al., 2013; 2016; Singh et al., 2014; Wink et al., 2014). The OACIS demonstrated high inter-rater reliability, along with cross-culture reliability (Choque Olsson & Bölte, 2013).

Vineland Adaptive Behavior Scale (VABS, Sparrow, Balla, and Cicchetti 1984). The VABS is a parent interview initially designed to examine functional age-appropriate everyday skills in four developmental domains (i.e., Communication, Socialization, Daily Living Skills, Motor Skills). The VABS provides an Adaptive Behavior Composite Score, which is based on the four domain scores for children up to age 5. For older children the composite score is based on three domains, excluding Motor Skills. The instrument's items are scored 0 (behavior not performed), 1 (performed sometimes) or 2 (performed regularly). The VABS was administered by a post-doc at weeks 0, 12, and 24. Raw scores were used. Past research showed that the VABS is a reliable and valid measure (e.g., Frye, DeLatorre et al., 2013; Frye, Melnyk et al., 2013; Sparrow, Cicchetti, Balla, 2005).

Child Measures

Expressive Vocabulary Test – Second Edition (EVT-2; Williams 2007). The EVT-2, administered by a master's level psychologist supervised by a post-doc at weeks 0 and 12, is an individually administered, norm-referenced instrument that assesses expressive vocabulary and word retrieval for children and adults. It is available in two parallel forms. Each form contains example items and 190 test items arranged in increasing difficulty. Raw scores were used. Past research demonstrated that the EVT-2 is a valid vocabulary test (e.g., Gray, Plante, Vance, and Henrichsen, 1999; Restrepo et al., 2006).

Peabody Picture Vocabulary Test (PPVT; Dunn & Dunn 2007). The PPVT-4 scale, administered by a master's level psychologist supervised by a post-doc at weeks 0 and 12, is a norm-referenced, wide-range instrument for measuring the receptive auditory vocabulary of children and adults. The PPVT is available in two parallel forms that are administered individually. Raw scores were used. Past research showed that the PPVT is a reliable and valid measure (e.g., Bracken, Prasse, and McCallum, 1984; Campbell, 1998). However, Restrepo et al. (2006) found that the EVT-2 was a better assessment for children's vocabulary skills than PPVT; especially for those children with low income and diverse backgrounds.

Statistical Analyses

In this small-sample pilot study, we emphasize effect sizes more than statistical significance, but did not neglect testing. The statistical tests using IBM SPSS Statistics 24 and R were two-tailed and $p \leq 0.05$ suggested statistical significance for the primary outcome of overall symptom improvement on the OACIS. The nine symptom-specific sub-items of the OACIS and the secondary questionnaires measuring different domains of functioning were exploratory and were tested without Bonferroni correction in this pilot study.

To test the two hypotheses introduced above, the scores on the OACIS-I (with overall symptom improvement representing the primary outcome) were analyzed using mixed-effects linear models. Estimating mixed effect linear models with a random intercept for participant; time, the treatment group as well as the treatment group-by-time interaction served as the fixed effects. Due to the small sample size ($N = 15$), we focused on effect sizes measured by Cohen's *d*, whereby values of 0.2, 0.5 and 0.8 were interpreted as suggesting small, medium, and large effects, respectively.

RESULTS

Demographics

Table 1 summarizes the demographics and other baseline characteristics of the study participants. There were initially no systematic differences between the placebo and cholesterol groups. Table 1 further illustrates that during the RCT, the cholesterol group was not more frequently affected by adverse events than the placebo group. Nevertheless, the cholesterol group had across most domains nominally higher baseline severity ratings (measured with the OACIS-S, see table 3) than the placebo group.

<<insert Table 1 about here>>

Primary Analyses: improvement in ASD symptomatology

Table 2 provides an overview of the mean ratings for overall symptoms of ASD and the nine domain-specific symptoms assessed by the OACIS-I. Note that the scale of the *OACIS-I* ranges from 1 (very much improved) to 8 (very much worse), wherein a rating of a 5 indicates no change. Thus, the lower the OACIS-I ratings, the bigger the improvement in symptoms of ASD: 4 = minimally improved; 3 = definitely improved; 2 = much improved. The last two columns of

table 2 denote the Cohen's *d* effect sizes for the RCT and open extension, respectively. Negative values in the RCT (baseline to week 12) indicate that the cholesterol group improved more than the placebo group.

<<insert Table 2 about here>>

The first row of table 2 represents the primary result summarizing the improvement in overall ASD symptoms for each group. The cholesterol group improved slightly (and non-significantly) more (*M = 3.50, SD = 1.07*) than the placebo group (*M = 3.67, SD = 1.21*) by the end of the RCT [*F = 0.087, df = 39.411, p = 0.770, Cohen's d = -0.15*]. Responders (participants with an OACIS-I score lower than 4 at the end of the RCT) were 63% (5 of 8) of the cholesterol group, compared to 43% (3 of 7) of the placebo group. After receiving cholesterol supplements in the open extension, the placebo group "caught up" to the cholesterol group, improving non-significantly more than the cholesterol group [*F = 0.148, df = 2.551, p = 0.148, Cohen's d = 0.48*] during the open extension. Aggregating over both groups, the correlation between the change in blood cholesterol level and the size of improvement (measured by the OACIS-I) from baseline to week 24 was small and nonsignificant [*r = -0.2575, t = -0.705, df = 7, p = 0.504*]. The cholesterol level of the placebo group increased by 15 mg/dL, from 118 at baseline to 133 at the end of open extension (week 24), while the cholesterol level of the cholesterol group increased by 25 mg/dL, from 108 at baseline to 133 at week 24 (nonsignificant difference; note that most week 12 labs were lost en route to the remote lab). Combining the examination of the RCT and open extension, the primary results can be summarized as follows:

Both groups improved in overall ASD symptomology during the first 12 weeks, with the cholesterol group improving minimally (nonsignificantly) more than the placebo group. After obtaining open-

label cholesterol, the original placebo group improved twice as much as the cholesterol group in the open extension.

For OACIS-I specific symptoms during the RCT (table 2), the cholesterol group improved more than the placebo group by medium to large effect sizes (Cohen's $d > 0.5$) for the domains "nonverbal communication," and "anxiety and fear." A linear mixed-effects regression model for the RCT was estimated for each of the 9 OACIS domains. We did not find any significant treatment group x time interactions.

In the open extension, the effect sizes suggest medium to large improvements within the original placebo group for the domains "social interaction," "aberrant behavior," "hyperactivity and inattention," and "anxiety and fear." Without corrections for multiple testing in these exploratory analyses, we found a significant effect for social interaction [$F = 7.170$, $df = 8.789$, $p = 0.026$]. The effects of cholesterol on domain-specific ASD symptoms measured with the OACIS, can be summarized as follows:

In the RCT, the cholesterol group improved more than the placebo group in 5/9 domains of specific ASD symptoms, with Cohen's d = -0.64 for nonverbal communication. In the 12-week open extension, the group originally assigned to placebo experienced more improvements than the continuing-cholesterol group in most domains of specific ASD symptom, particularly in OACIS-I social interaction.

Exploratory Analyses: Beneficial effects on Communication Skills

Because the cholesterol group showed more improvement in the randomized phase than the placebo group in nonverbal communication by a medium effect (Cohen's $d = -0.64$) on the

OACIS-I, we explored communication in secondary scales: VABS; EVT, and PPVT (see Figure 1).

<<insert Figure 1 about here>>

During the RCT phase the cholesterol group improved significantly more than the placebo group on the PPVT-4 receptive language [$F = 5.200$, $df = 11$, $p = 0.040$] (Figure 1), but not on either the EVT [$F = 1.00$, $df = 9.8$, $p = 0.350$] nor the VABS [$F = 0.083$, $df = 10.112$, $p = 0.779$]. Across both groups, there was a moderate nonsignificant correlation between the change from baseline to week 24 in cholesterol level and the size of improvement on OACIS-I verbal communication [$r = 0.3632$, $t = 1.031$, $df = 7$, $p = 0.337$] and OACIS-I nonverbal communication [$r = 0.5869$, $t = 1.918$, $df = 7$, $p = 0.097$]. In sum, the findings for "communication" can be summarized as follows:

Evidence from the OACIS-I as well as from specialized measures for the assessment of the domain of communication suggests beneficial effects of cholesterol on the communication skills of children with ASD that only occasionally reach significance at this sample size.

<<insert Table 3 about here>>

Exploratory Analyses: Beneficial effects on Anxiety/Fear

The results in section A suggested that cholesterol may provide benefits in anxiety/fear. Specifically, the Cohen's *d* in table 2 suggested a medium-to-large effect compared to placebo in the RCT (Cohen's $d = -0.67$), and a large effect (Cohen's $d = 0.92$) for the improvement of the original placebo group relative to the continuing-cholesterol group in the open extension. Figure 2 illustrates that both groups experienced less anxiety/fear over the course of the study. Aggregating over both groups, there was a modest nonsignificant correlation between the change

in cholesterol level and the size of improvement from baseline to week 24 [*r = -0.3274, t = -0.917, df = 7, p = 0.390*].

<<insert Figure 2 about here>>

Figure 2 and the Cohen's *d* effect sizes in table 2 suggest that the cholesterol group improved more than the placebo group during the RCT, and the placebo group caught up during the open extension. However, these group differences were statistically nonsignificant [*F = 0.569, df = 19.333, p = 0.460* for the RCT, *F = 2.098, df = 8.281, p = 0.184* for the open extension]. In sum, one can conclude:

Large effect sizes for anxiety/fear in response to cholesterol are not significant at this sample size.

DISCUSSION

This pilot RCT with open-label extension explored effects of cholesterol on ASD symptoms in children with cholesterol levels below the 5th percentile of normal for age and sex. With only 15 participants, we did not expect statistical significance, but emphasize effect sizes. In the RCT, the primary outcome, overall improvement on the OACIS-I, showed nominally higher response rates (63% vs. 43%) for cholesterol than for placebo and nominally higher general improvement (Cohen's *d = -0.15, p = 0.770*). Specific ASD symptoms showed larger effects. The cholesterol group showed more improvement than placebo in 5 of 9 specific symptoms, including medium effects in the domains nonverbal communication (Cohen's *d = -0.64, p = 0.572*) and anxiety/fear (Cohen's *d = -0.67, p = 0.460*). The mean of the 5 effect sizes by which cholesterol surpassed placebo was Cohen's *d* = 0.44; the mean of the 4 effect sizes by which placebo surpassed cholesterol was Cohen's *d* = 0.32. Moreover, cholesterol showed significantly more improvement than placebo in PPVT-4 receptive language (*p=0.04*); although not on either the EVT [*F = 1.00, df = 9.8, p = 0.35*] nor the VABS [*F = 0.083, df = 10.112, p = 0.779*]. Given the mean age of both

groups (around 6 years) and the limited vocabulary of children (especially those with autism) at this age, a receptive vocabulary test (PPVT) may provide a more appropriate measure of their communication skills than an expressive vocabulary test (EVT).

After obtaining cholesterol in the open extension, participants in the original placebo group caught up on most OACIS scales. They improved nonsignificantly more during the open-label 12 weeks than participants given cholesterol throughout the study in overall ASD symptomatology (Cohen's *d = 0.48, p = 0.148*) and significantly more in social interactions (Cohen's *d = 1.17, p = 0.026*).

These encouraging pilot results were obtained with neither theoretical nor empirical evidence of safety concerns. Giving a child with abnormally low cholesterol the equivalent of two eggs a day appears *prima facie* safe, and the recording of adverse events revealed nothing to challenge that assumption. Although this potential treatment would be applicable to less than 10% of children with ASD (we found 8% of those screened to have cholesterol below the 5th percentile for age and sex), it could be an important addition to treatment options, given the paucity of treatments for ASD. Moreover, lower cholesterol in children with ASD has been associated with higher incidences of fungal infections and gastrointestinal disorders, including chronic diarrhea and malabsorption of key nutrients (Tierney et al., 2006).

Limitations

The sample size of 15 is obviously very small for a double-blind, randomized, parallel-group study, making type 2 errors likely. Another limitation could be the use of the OACIS (i.e., overall symptom improvement) as an instrument for measuring improvement. It could be that the OACIS was not a sufficiently sensitive instrument. However, there has not been any established instrument for measuring outcome on core ASD symptoms thus far. Further, the dose, although consistent with what is used for SLOS, may have been suboptimal for non-SLOS ASD. We ruled

out SLOS by standard tests. Finally, the duration of treatment may not have been adequate to show full effects: The graphs suggest continued improvement of the cholesterol group during open-label extension despite the "catch-up" by the group originally assigned placebo.

In sum, although most of the benefit of cholesterol compared to placebo was statistically nonsignificant at this small sample size, the effect sizes suggest that cholesterol supplementation for ASD with low cholesterol should be studied further. There does not seem to be a great risk to supplementing with the amount of cholesterol in two eggs for those with cholesterol below the 5th percentile unless they are allergic to eggs, but future studies should cautiously explore larger doses, perhaps titrating against cholesterol blood levels, as well as longer duration.

DECLARATIONS

Ethics approval. The research was approved by The Ohio State University's Institutional Review Board (IRB). The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Consent to participate. Informed consent from parents and assent (if the child was capable) from participants was obtained using forms and procedures approved by the Ohio State University IRB.

Consent to publish. The authors affirm that human research participants provided informed consent for the use and share of their de-identified information for future research and publication.

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Tables

Table 1. Sample Characteristics, Demographics, and Adverse Events throughout the RCT.

	Placebo Group (N = 7)		**Cholesterol Group (N = 8)**	
Baseline Characteristics	**mean**	**SD**	**mean**	**SD**
Age (in years)	6.30	2.30	6.90	3.00
Gender (females, males)	2, 5		1, 7	
Race	1 African-American 6 Caucasian		1 African-American 1 Multi-Racial 6 Caucasian	
Weight (in kg)	23.97	8.81	22.90	6.15
Height (in cm)	118.88	11.76	119.60	16.45
Cholesterol level at baseline (in mg/dL)	117.71	7.30	107.75	19.22
ASD symptom severity at baseline (measured with OACIS-S)	4.20	1.10	5.17	1.47
Communication at baseline (raw score measured with EVT)	52.00	25.61	54.00	26.76
Communication at baseline (raw score measured with PPVT)	52.50	21.52	64.13	35.13
BMI	16.50	2.83	16.13	1.76
IQ	58.00	26.69	75.00	30.47
Adverse Events (During RCT)	**Frequency of event**	**No. of subjects**	**Frequency of event**	**No. of subjects**
General-Constitutional	2	1	2	2
Clinically Significant Abnormal Labs	0	0	0	0
Eyes/Ears/Nose/Throat	1	1	4	3
Cardiac	0	0	0	0
Respiratory	1	1	5	2
Gastrointestinal	11	4	5	3
Renal/Urinary/Reproductive	2	1	0	0
Neurological	5	3	0	0
Behavioral	2	2	0	0
Skin	3	3	1	1
Musculoskeletal	0	0	0	0
Totals	*27*	*16*	*17*	*11*

Mean ratings with respective standard deviations (SDs). Missing values were excluded from these summary statistics (without excluding entire participants). Adverse events are illustrated for baseline throughout week 12 (e.g., during random clinical trial phase (RCT)).

Table 2. Primary outcome: Ohio Autism Clinical Impression-Improvement Scale (OACIS-I) - Mean Ratings, standard deviations, and between-group effect sizes for RCT and open extension.

Domain	**Week**	**Cholesterol Group**		**Placebo Group**		**Cohen's d (Cholesterol-Placebo)**	
		mean	**SD**	**mean**	**SD**	**End RCT** (from BL to week 12)	**End Open extension** (from week 12 to 24)
overall ASD symptomatology	0	5.00	0.00	5.00	0.00		
	12	3.50	1.07	3.67	1.21	-0.150	
	24	3.00	1.41	2.60	0.89		0.483
Problems with Social Interactions	0	5.00	0.00	5.00	0.00		
	12	3.50	1.05	3.80	1.30	-0.257	
	24	3.17	1.17	2.40	0.55		1.170*
Aberrant Behavior	0	5.00	0.00	5.00	0.00		
	12	4.67	1.86	4.60	1.14	0.044	
	24	4.17	1.33	2.80	1.48		0.924
Repetitive/ Ritualistic Behavior	0	5.00	0.00	5.00	0.00		
	12	4.17	1.47	4.60	1.67	-0.275	
	24	3.00	1.90	3.60	0.89		-0.115
Verbal Communication	0	5.00	0.00	5.00	0.00		
	12	3.50	1.52	4.00	1.00	-0.380	
	24	2.67	1.37	2.60	0.89		0.493
Nonverbal Communication	0	5.00	0.00	5.00	0.00		
	12	4.17	0.75	4.60	0.55	-0.643	
	24	3.33	1.37	3.80	1.30		-0.030
Problems with Hyperactivity/ Inattention	0	5.00	0.00	5.00	0.00		
	12	4.67	1.03	4.20	1.30	0.406	
	24	3.83	1.47	2.60	1.52		0.508
Severity in Sensory Sensitivity	0	5.00	0.00	5.00	0.00		
	12	4.33	1.03	4.20	1.30	0.112	
	24	3.67	1.51	4.40	0.89		-0.694
Problems with Anxiety and Fear	0	5.00	0.00	5.00	0.00		
	12	4.00	2.00	5.20	1.48	-0.671	
	24	4.33	1.51	3.80	2.17		0.925
Problems with Restricted/ Narrowed Interests	0	5.00	0.00	5.00	0.00		
	12	4.50	0.84	3.80	1.10	0.726	
	24	3.67	1.51	2.80	1.48		0.114

The lower the rating, the bigger the improvement in Autism spectrum disorder symptomology. Rating of 5 means no change from baseline. The last two columns represent the Cohen's d effect sizes for independent samples. Subtraction of Placebo group from Cholesterol group. Negative (positive) Cohen's d values indicate that the Cholesterol group improved more (less) than the Placebo group. To estimate covariance for Cohen's d, only pairwise complete observations were used. Interpretation of the effect sizes: 0.2 = small, 0.5 = medium, 0.8 = large. *$p \leq 0.05$, based on mixed-effects linear models. RCT: random clinical trial phase (from baseline to week 12).

Table 3. Secondary/exploratory outcome measures - Mean Ratings, standard deviations and between-group effect sizes for RCT and open extension.

Domain	**Week**	**Cholesterol Group**		**Placebo Group**		**Cohen's d (Cholesterol-Placebo)**	
		mean	**SD**	**mean**	**SD**	**End RCT** (from BL to week 12)	**End Open extension** (from week 12 to 24)
Secondary/Exploratory Outcome: Ohio Autism Clinical Impression-Severity Scale (OACIS-S)							
overall ASD symptomatology	0	5.17	1.47	4.20	1.10		
	12	5.17	1.47	3.83	1.33	0.264	
	24	4.80	1.30	4.00	1.23		-0.427
Problems with Social Interactions	0	5.50	1.23	4.80	0.84		
	12	5.33	1.21	4.33	1.03	0.267	
	24	5.00	1.00	4.20	1.10		-0.191
Aberrant Behavior	0	3.83	1.17	3.20	1.79		
	12	3.67	1.21	2.83	1.84	0.135	
	24	3.60	1.52	2.20	1.10		0.423
Repetitive/ Ritualistic Behavior	0	4.50	1.52	3.00	1.58		
	12	4.33	1.37	2.67	1.63	0.106	
	24	3.40	0.89	2.80	1.64		-0.801
Verbal Communication	0	5.17	1.60	5.00	1.23		
	12	5.00	1.41	4.67	1.21	0.122	
	24	4.40	1.52	4.60	1.34		-0.370
Nonverbal Communication	0	4.50	1.87	4.80	1.30		
	12	4.50	1.87	4.33	1.63	0.268	
	24	3.80	1.64	4.60	1.34		-0.647
Problems with Hyperactivity/ Inattention	0	5.00	0.89	5.00	0.71		
	12	5.00	0.89	4.50	1.23	0.466	
	24	4.40	0.89	4.00	0.71		-0.124
Severity in sensory Sensitivity	0	4.67	0.82	3.40	1.67		
	12	4.50	0.84	3.00	1.79	0.165	
	24	4.00	0.71	3.40	1.67		-0.701
Problems with Anxiety and Fear	0	2.83	1.72	2.80	2.17		
	12	2.83	1.60	3.17	1.72	-0.222	
	24	3.00	1.23	2.40	1.14		0.794
Problems with restricted/ narrowed interests	0	5.00	1.67	3.40	0.89		
	12	5.00	1.67	2.83	0.98	0.415	
	24	3.40	1.67	2.60	0.55		-1.101

Continued on next page.

Table 3 *(continued)*

Domain	Week	Cholesterol Group		Placebo Group		Cohen's d (Cholesterol-Placebo)	
		mean	SD	mean	SD	**End RCT** (from BL to week 12)	**End Open extension** (from week 12 to 24)
Secondary/Exploratory Outcome: Vineland Adaptive Behavior Scale (VABS)							
Daily Living Skills	0	31.33	7.01	33.20	10.87		
	12	31.17	5.71	32.80	10.69	0.029	
	24	35.60	3.05	33.20	9.09		0.594
Socialization	0	28.00	7.80	31.40	7.30		
	12	30.00	5.25	30.80	8.44	0.379	
	24	30.80	3.03	32.80	9.42		-0.172
Communication	0	29.83	11.86	28.40	8.11		
	12	31.17	8.18	30.40	9.89	-0.073	
	24	36.60	5.98	31.00	9.72		0.599
Secondary/Exploratory Outcome: Expressive Vocabulary Test (EVT)							
Raw score	0	54.00	26.76	52.00	25.61		
	12	53.00	23.58	48.83	20.17	0.098	
Secondary/Exploratory Outcome: Peabody Picture Vocabulary Test (PPVT)							
Raw score	0	64.13	35.13	52.50	21.52		
	12	72.00	40.74	52.33	24.55	0.234*	

For OACIS-S: Lower scores are better. Negative (positive) Cohen's d values indicate that the Cholesterol group improved more (less) than the Placebo group. For VABS/EVT/PPVT: Higher scores are better. Negative (positive) Cohen's d values indicate that the Cholesterol group improved less (more) than the Placebo group. To estimate covariance for Cohen's d, only pairwise complete observations were used. Interpretation of the effect sizes: 0.2 = small, 0.5 = medium, 0.8 = large. *$p \leq 0.05$, based on mixed-effects linear models. RCT: random clinical trial phase (from baseline to week 12). ASD: autism spectrum disorder.

Figures

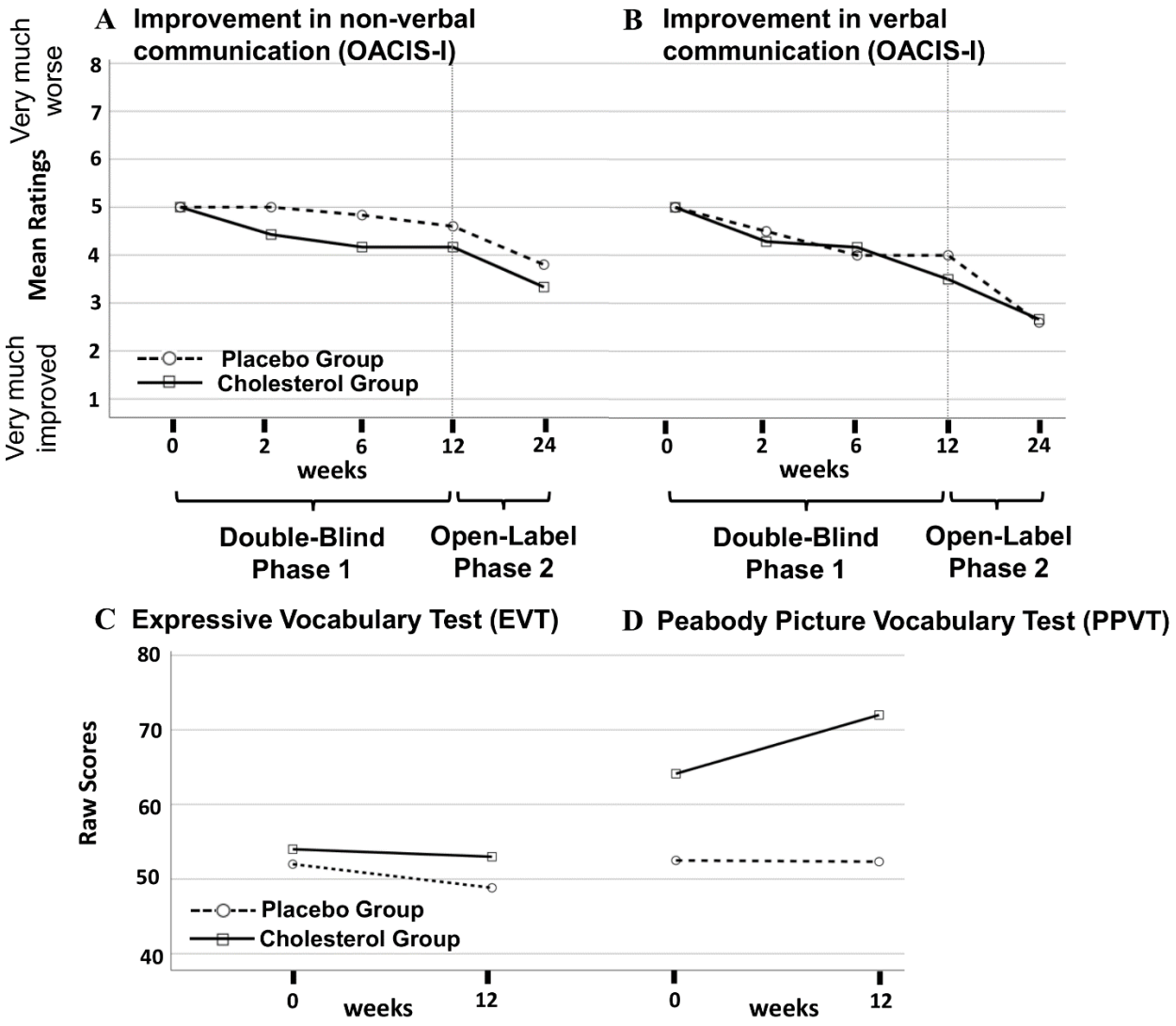

Figure 1. *Specific measures of communication skills – top 2 panels (A, B) represent mean ratings measured by OACIS-I (lower ratings are better). Bottom 2 panels (C, D) represent raw scores during RCT measured by PPVT and EVT (higher ratings represent improvement).*

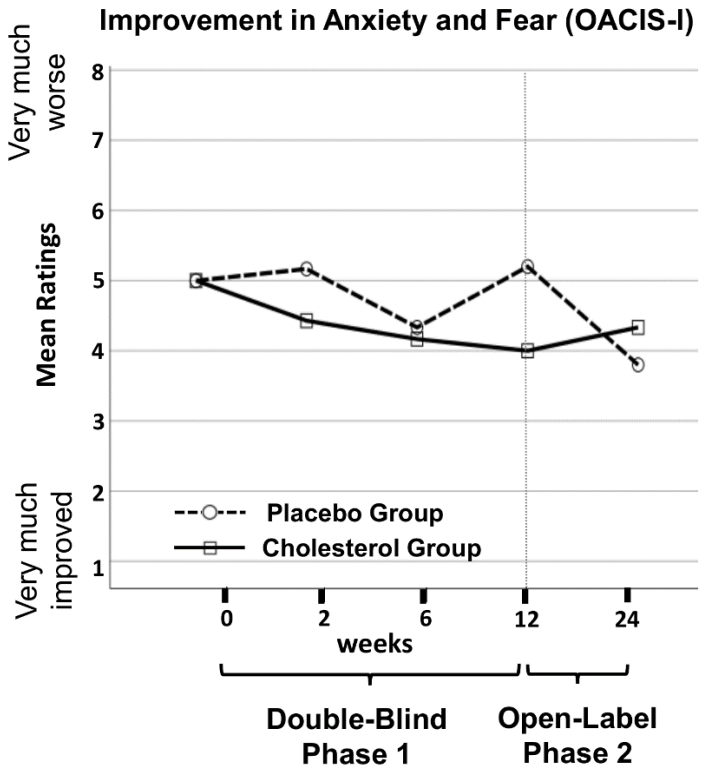

Figure 2. *Improvement in Anxiety and Fear (measured by OACIS-I). The lower the rating, the bigger the improvement. A rating of 5 means no change from baseline.*