RANDOMIZED CONTROLLED TRIAL OF MATERNAL CHOLINE SUPPLEMENTATION: EFFECTS ON INFANT INFORMATION PROCESSING SPEED

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

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December 2021

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ABSTRACT

Despite mounting evidence for the critical role of choline in neurodevelopment, nearly 90% of pregnant women consume choline at levels below the Adequate Intake (AI). The preponderance of this evidence for increasing maternal choline intake comes from studies with rodent models, but a recent supplementation trial manipulated the choline intake of pregnant women with a controlled diet and provided evidence that maternal choline supplementation (MCS) improves offspring cognitive functioning during infancy and at age 7. However, the only two experimental studies examining offspring functional outcomes when a choline supplement was delivered alongside a woman's usual diet provided conflicting and inconclusive results. Therefore, it remains unclear whether widespread MCS would be an effective public health strategy for pregnant women to optimize offspring cognitive development. This thesis reports results from a double-blind placebo-controlled trial in which second-trimester pregnant women were randomized to consume a supplement of either 550 mg (n=13) or 25 mg (n=12) of choline daily while remaining on their usual diets. The Visual Expectation Paradigm (VExP) task was administered when offspring were 5, 7, 10, and 13 months of age. This visual attention task is designed to measure the latency of saccadic eye movements an infant makes toward stimuli while viewing a sequence of animations appearing on the left or right side of a monitor. These saccades are categorized as reactive or anticipatory. Infant information processing speed, a predictor of adolescent IQ, was determined by an infant's mean saccade reaction time (RT) while the percentage of anticipatory saccades provided an index of infant visuospatial memory. Data analysis, using a pre-specified linear mixed-effects regression model, revealed that mean RT for infants in the 550 mg/d choline group was 22.3 ms faster than those in the control group (P=0.047). Percent anticipation did not differ between groups. These findings demonstrate that

supplementing a pregnant woman's diet with choline significantly improves infant information processing speed, a capacity foundational to the development of higher-level cognitive abilities.

BIOGRAPHICAL SKETCH

Jesse grew up in the small town of Chelsea, Vermont, where she attended elementary and high school, enjoyed playing various sports and met her husband and many friends. She studied Dietetics, Nutrition, and Food Sciences at the University of Vermont (UVM), where she had the opportunity to work on various nutrition-related research projects as an undergraduate research assistant and explore the world of clinical dietetics at the University of Vermont Medical Center. She completed her Dietetic Internship at Cornell University in 2021, during which she fell in love with neonatal medical nutrition therapy and subsequently became a Registered Dietitian Nutritionist. Jesse joined the Strupp lab at the beginning of her graduate studies and has since worked with Drs Strupp and Canfield to investigate the effect of maternal choline supplementation on offspring cognitive and affective outcomes. When she is not in the classroom, laboratory, or hospital, Jesse enjoys traveling and cooking with her lifelong best friend and husband, spending time with her family, running, skiing, or playing tourist in her home state.

ACKNOWLEDGMENTS

I am tremendously grateful to my advisor Dr. Barbara Strupp for her boundless support for my research, academics, and overall well-being. Barb's guidance and mentorship have allowed me to discover my passions and overcome unexpected adversities. I am also indebted to Dr. Richard Canfield, without whom this thesis would not have been possible. Rick's dedication to excellence in both research and advising is inspiring. I owe my deepest gratitude to Kara Beckman, my fellow lab-mate, an invaluable resource and excellent therapist, and Elizabeth Feathers, our lab manager and great friend, who worked tirelessly behind the scenes to make our lives easier. I am also incredibly appreciative of the outstanding CholLab undergraduate researchers. Finally, I would like to thank my husband, family, and friends for their humor, tough love, and endless support.

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LIST OF ABBREVIATIONS

Term	Abbreviation
Acetylcholine	ACh
Adequate Intake	AI
American Society for Parenteral and Enteral Nutrition	ASPEN
Anticipatory Saccade Latencies	ANTL
Dietary Guidelines for Americans	DGA
Dietary Reference Intake	DRI
Docosahexaenoic Acid	DHA
Eicosapentaenoic Acid	EPA
Estimated Average Requirement	EAR
Executive Function	EF
Maternal Choline Supplementation	MCS
Reaction Time	RT
Recommended Dietary Allowance	RDA
Registered Dietitian Nutritionist	RDN
Phosphatidylcholine	PC
Phosphatidylethanolamine N-methyltransferase	PEMT
Single Nucleotide Polymorphism	SNP
Tolerable Upper Limit	UL
Total Parenteral Nutrition	TPN
Visual Expectation Paradigm	VExP

INTRODUCTION

Prenatal nutrient deficits, especially when they occur during periods of rapid brain growth and maturation, have a substantial and lasting impact on a child's neurodevelopmental trajectory[1,2]. In recent years, choline has been identified as a nutrient of key concern for protecting and optimizing neurodevelopment throughout the life course[3–6]. Choline is considered an essential nutrient because, although humans synthesize endogenous choline via the hepatic phosphatidylethanolamine N-methyltransferase (PEMT) pathway, most individuals must consume dietary choline to prevent deficiencies[7]. Pregnancy demands an extraordinary supply of choline which plays a central role in brain development, tissue expansion, and placental function. Notably, animal models of prenatal choline deficiency find perturbed progenitor cell development and irreversible cognitive deficits in the offspring. Meanwhile, a vast body of rodent research demonstrates that increased choline availability during the prenatal period protects the fetal brain from damage associated with various neuropathologies, and supplementation of choline to the maternal diet improves offspring attention and memory. Though few human studies have explored this, recent evidence from a small controlled feeding trial suggests that the cognitive benefits of maternal choline supplementation (MCS) seen in rodent models may also translate to human infants, with benefits persisting to early childhood[8–10]. Therefore, failure to supply adequate choline to the developing fetus could threaten optimal brain and cognitive health throughout life.

Importance of Maternal Choline Intake for Fetal Development

Although the mechanism by which maternal choline intake can modulate offspring neurodevelopment remains incompletely understood, converging evidence suggests a combination of factors are involved, including the availability of constituents critical for cell

membranes, neurotransmitters, and epigenetic modifications (**Figure 1**). At the cellular level, choline's metabolites, phosphatidylcholine (PC) and sphingomyelin, are critical for efficient nerve signal transduction, myelination of nerve axons, membrane biogenesis, and cell division[5,7]. Choline is a central component of acetylcholine (ACh), a major neurotransmitter involved in regulating neuronal proliferation and maturation, with considerable roles in learning, memory, and neuroplasticity[3,6,7,11]. Finally, the methyl donor betaine, produced through the irreversible oxidation of choline, modifies methylation of brain DNA and histones, resulting in lasting epigenetic modifications of many genes, including some known to be involved in learning and memory[6,7,12–14].

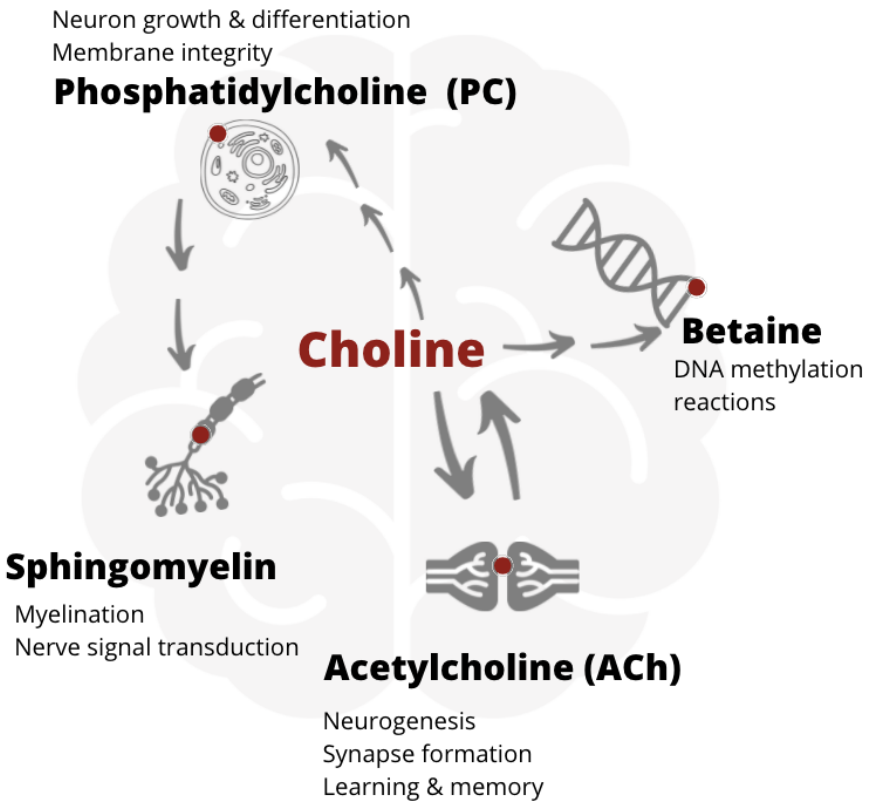

Figure 1. The effects of choline on neurodevelopment via the metabolites of choline. Although each of these pathways provides a plausible mechanism for lasting offspring cognitive effects of MCS, the extent to which each of these different pathways mediates these effects remains to be determined.

In light of these vital functions of choline and growing empirical evidence, both the American Academy of Pediatrics and American Medical Association have concluded that inadequate prenatal choline availability is detrimental to a child's cognitive health[15,16]. This statement followed by more than 20 years the 1998 the Food and Nutrition Board of the Institute of Medicine report which established the Dietary Reference Intake (DRI) for choline. However, because at that time there was insufficient evidence from human randomized placebo-controlled trials to establish the more rigorous Estimated Average Requirements (EAR) and Recommended Dietary Allowances (RDA), choline dietary recommendations were set as Adequate Intake (AI). Notably, the empirical basis for the current AI levels that were set in 1998 was a single study that provided a value for the minimal daily choline intake required to prevent liver dysfunction in men. These values were then extrapolated to women, other age groups, and various life-stages including pregnancy and lactation. This work resulted in setting the choline AI for pregnancy at 450 mg/d. In the more than two decades since the AI was established, a growing body of research has suggested that this poorly grounded AI may not fully meet the demands of the developing fetus.

During pregnancy, a period of rapid tissue growth and nervous system development, the physiological demand for choline increases substantially. This increased demand is matched by a surge of estrogen which upregulates the maternal PEMT pathway[17]. Notably, the fetus cannot produce sufficient choline on its own, requiring it to draw on the mother's endogenously produced and diet-derived choline supply to meet its own needs (see **Figure 2**). Human studies demonstrate that the significant increase in fetal brain choline concentrations coincides with a marked depletion of the maternal choline pool observed during gestational weeks 18 and 40[18,19]. Critically, this depletion was not prevented in women consuming choline at double the AI level.

Furthermore, a newborn's plasma choline concentration is 6 to 7 times greater than the mother's[20], which likely reflects a need to make the nutrient available in adequate amounts to support cell growth. Presumably, a chronic depletion of a woman's choline pools throughout pregnancy will diminish choline supplies available for her developing fetus and thereby compromise fetal neurodevelopment. The fact that intake at double the AI appears to not meet the demands of pregnancy strongly suggests that the AI itself is inadequate to meet both maternal and fetal demands. Therefore, it is concerning that recent surveys find only about 8% of pregnant women consume choline at even the AI level and that the average intake among this population is only 70% of the AI[21].

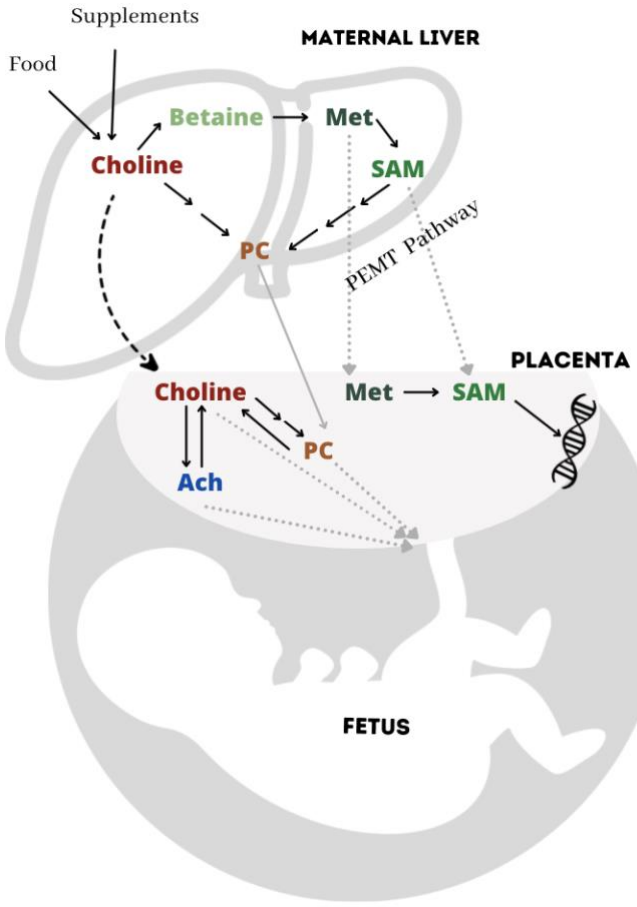

Figure 2. Transfer of choline metabolites from the maternal liver to the fetus through the placenta. The developing fetus draws on the maternal choline supply, derived from diet, supplementation, and the estrogen-induced PEMT pathway. Choline metabolites synthesized in the maternal liver are transferred to the placenta for transport to the fetus. Ach = Acetylcholine; PC = Phosphatidylcholine; Met = Methionine; SAM = S-adenosylmethionine; PEMT = Phosphatidylethanolamine N-methyltransferase. Adapted from Jiang et al. 2014.

Maternal Choline Intake and Offspring Neurodevelopment:

Rodent Studies

Robust findings from animal studies reinforce the notion that maternal choline intake affects fetal brain development and lifelong offspring cognitive functioning. Specifically, altering the choline intake of dams and sows results in lasting structural[22–28], neurochemical[3,5,29–32], and electrophysiological[33,34] alterations in the offspring's brain. These changes are prominent in the basal forebrain region, which has cholinergic projections to the entire cortex and hippocampus, two areas implicated in learning and memory, attention regulation, and decision-making[3,27,29,35]. And, perhaps most importantly, supplementing the diet of pregnant rats with additional choline (4-5 times the amount in normal chow) significantly improves offspring memory and attention across the lifespan[5,22,23,36–39].

Furthermore, there is compelling evidence that maternal choline supplementation during pregnancy can offer protection against a variety of offspring neuropathologies. A vast body of rodent research demonstrates that increased choline availability throughout gestation and during the prenatal period protects the offspring's brain from damage associated with epilepsy[40–42], fetal alcohol syndrome[43–47], Rett syndrome[48–51], and Down syndrome[24,25,37,52–55]. More recent rodent studies have also highlighted the role of maternal choline supplementation (MCS) in reducing cognitive dysfunction seen in rodent models of Alzheimer's disease[52,56] and maternal stress.[57]

Human Studies

Evidence for the neuroprotective role of choline in humans is scarce, but supported by observational studies showing maternal choline intake is inversely associated with risk of adverse pregnancy outcomes including neural tube defects[58,59], preterm delivery[60], and risk of disability and cognitive impairment in children exposed in utero to marijuana[61] or severe infections[62]. More compelling support for this translation comes from two randomized controlled

trials in which MCS mitigated the adverse effects of prenatal alcohol exposure on fetal neurodevelopmental outcomes in infants[63,64].

Maternal Choline Intake and Offspring Cognition:

Observational Human Studies

Although research evaluating the effects of maternal choline intake on cognitive functioning in healthy offspring is limited, a few recent studies support the notion that these effects may also translate to humans. Among the observational studies, Boeke et al. found a negative correlation between lower second-trimester maternal choline intake and a child's performance on a standardized memory assessment at age seven[65]. Similarly, Wu et al. found a positive association between maternal plasma free choline levels at 16 weeks gestation and Bayley Scales of Infant Development-III test scores at 18 months[66]. Importantly, these studies cannot address the potential benefits of higher maternal choline intakes because a majority of participants in the former study had choline intakes below the AI and plasma choline, the biomarker used in the latter study, is homeostatically regulated and therefore lacks validity as an indicator of choline intake[67]. Two other observational studies found no correlation between maternal choline intake and cognitive functioning, although measurement errors in estimated dietary choline intake and the use of non-fasting choline metabolite data may account for these mixed results[68,69]. Ultimately, because average dietary choline intakes are low, observational studies cannot address the potential benefits of maternal choline at intakes substantially greater than the AI, as has been found consistently in animal studies.

Human Choline Supplementation Trials

To date, only three human supplementation studies have explored the hypothesis that maternal choline supplementation will improve offspring cognitive functioning in typically-developing infants, as has been seen in the rodent studies. In a randomized placebo-controlled

trial of phosphatidylcholine (PC) supplementation beginning at the 16th week of gestation, Ross et al. demonstrated a beneficial effect of supplementation on an indirect measure of infant attention— an electrophysiologic assessment of cerebral inhibition at the fifth postnatal week of life[70]. However, no difference was found at the 13th week of life. Results from the same group suggested that children of mothers consuming the PC supplement had reduced attentional disorders at 40 months of age, as measured by the Child Behavior Checklist, a parent-report of child behavior[71]. Yet, another study found no benefits of maternal PC supplementation (equivalent to 750 mg/d of choline) on offspring memory. However, procedural and analytic problems in the latter study raise questions about the validity of these null findings[72]. Consistent with evidence that intake at the AI might not meet fetal demands, a recent supplementation trial demonstrated that maternal choline intake above the AI level may produce more clearly significant cognitive benefits in offspring. In this small controlled feeding study, Caudill et al. reported that 930 mg/d of choline intake throughout the third trimester significantly improved infant processing speed as compared to intake at 480 mg/d. A follow-up study of these infants at age 7 conducted in the same lab as the present study revealed that offspring of mothers consuming the higher choline level had superior color-location memory[10] and sustained attention[9], as compared to children of mothers who had consumed only the AI. Importantly, this study demonstrated the efficacy of maternal choline supplementation, epidemiologically defined as the capacity of an intervention under ideal and controlled circumstances[72].

Although compelling, this small, controlled feeding study required participants to consume meals in the laboratory and follow a rigid diet, which is neither a feasible nor practical means of supplementation. This methodological limitation precludes the extension of the observed beneficial effects of MCS to children in real-world practice conditions where women

consume an *ad libitum* diet[73]. Moreover, the inconsistent results observed in the two studies in which choline supplements were added to women's usual diets add ambiguity to this issue[71,72]. Therefore, the question of whether a similar magnitude of superior task performance would be seen with choline supplementation alongside a woman's unrestricted diet, termed effectiveness in epidemiological trials, remains unanswered. Unlike a controlled diet, the nutrient composition of a usual diet varies between women and even within women from day-to-day, which may interact with choline metabolism or independently influence infant cognition. Despite preliminary evidence for the efficacy of choline intake on offspring cognitive outcomes, rigorously designed randomized controlled trials, employing practical supplementation methods and sensitive cognitive outcomes, are needed to determine the effectiveness of widespread MCS as a public health strategy.

Effects of Maternal Choline Supplementation on Infant Processing Speed: The Present Study

Addressing this critical gap in research, we conducted a confirmatory randomized placebo-controlled trial contrasting 550 mg/d and 25 mg/d choline supplementation to pregnant women alongside their usual unrestricted diets. We then administered a battery of cognitive assessments to their offspring throughout the first year of life. The present report analyzes findings from the Visual Expectation Paradigm (VExP), the same task administered in the controlled feeding study described above, which enables direct comparison of offspring task performance when MCS is delivered in a controlled diet versus added to an unrestricted diet. The VExP places demands on the child's attentional abilities and provides an index of infant processing speed and predicts attentional orienting speed and intelligence test scores in preadolescence [74–76].

The Role of Attention in Cognitive Developmental Cascades

In developmental terms, information processing speed reflects the performance of the attentional orienting network, a distinct network within the broader attentional system responsible for prioritizing stimuli by selecting a sensory modality or spatial location relevant to the stimulus for further processing, often prompting a shift in visual attention. Importantly, measures of infant attention and information processing efficiency are among the few outcomes shown to predict later cognitive functioning and academic achievement in typically-developing children[74–78]. This is thought to be due to a developmental cascade in which basic attentional abilities serve as a foundation for the development of higher-level cognitive abilities such as memory and reasoning. For instance, the ability to represent information as a memory first requires the brain to attend to the source of information and encode the information. Accordingly, faster orienting of attentional resources towards a stimulus implies faster processing and encoding of relevant information, allowing for greater efficiency of learning, such as vocabulary attainment and perceptual discrimination —processes likely at the core of the developmental cascade connecting infant information processing to intelligence and academic achievement later in life[78].

Despite rodent studies demonstrating the essential role of choline for optimal development of the attentional network, few interventions have adequately addressed this hypothesis in humans. Among the existing interventions that have examined the cognitive benefits of MCS, one relied on parent-reported behavioral ratings[71], likely resulting in measurement error, and the other required participants to consume study meals containing precise amounts of choline which precludes conclusions about the effectiveness of MCS[8,9]. Therefore, a central goal of our study was to determine whether supplementing a woman's

regular diet with choline benefits offspring information processing speed at a similar magnitude as when women consume a strictly controlled diet.

METHODS

Study Design & Participants

The present study leveraged an ongoing randomized, double-blind, controlled supplementation trial conducted at Cornell University. The supplementation trial (later referred to as the pregnancy study) was powered to assess the primary outcomes of maternal/fetal biomarkers of choline metabolism. Secondary outcomes included offspring cognition during infancy, genomic expression, and metabolomic profiling of plasma and placental tissue (ClinicalTrials.gov as NCT03194659). The current study (later referred to as the cognitive follow-up study) is an ancillary follow-up of offspring from the initial pregnancy study at 5-, 7-, 10- and 13-months postnatal age to assess effects on infant cognition, using pre-specified endpoints. The methods of the original trial are described in detail elsewhere[79].

In short, pregnant women were recruited at maternity clinics throughout Ithaca, NY, between October 2017 and April 2019. Women were eligible to participate in the pregnancy study if they were 21-40 years old, entering their second trimester of pregnancy, and had a pre-pregnancy BMI of <32 kg/m^2. Women who were using alcohol, recreational drugs, tobacco products, or medications that are known to affect liver and kidney function during pregnancy were excluded from the study at screening. Women with self-reported medical conditions or treatments associated with adverse effects on digestion and nutrient absorption or fetal development and infant cognition were also excluded from participation. Further, women with high baseline omega-3 fatty acid or choline intakes assessed through dietary questionnaires were

ineligible to participate. Any enrolled participants who developed pregnancy-related health complications (i.e. preeclampsia, miscarriage, gestational diabetes) during pregnancy were removed from the study. As illustrated in **Figure 3,** three participants- two from the treatment group and one from the control group- were excluded post-allocation due to the development of gestational diabetes. Eligibility criteria for the cognitive follow-up study were the same as those used in the initial pregnancy study.

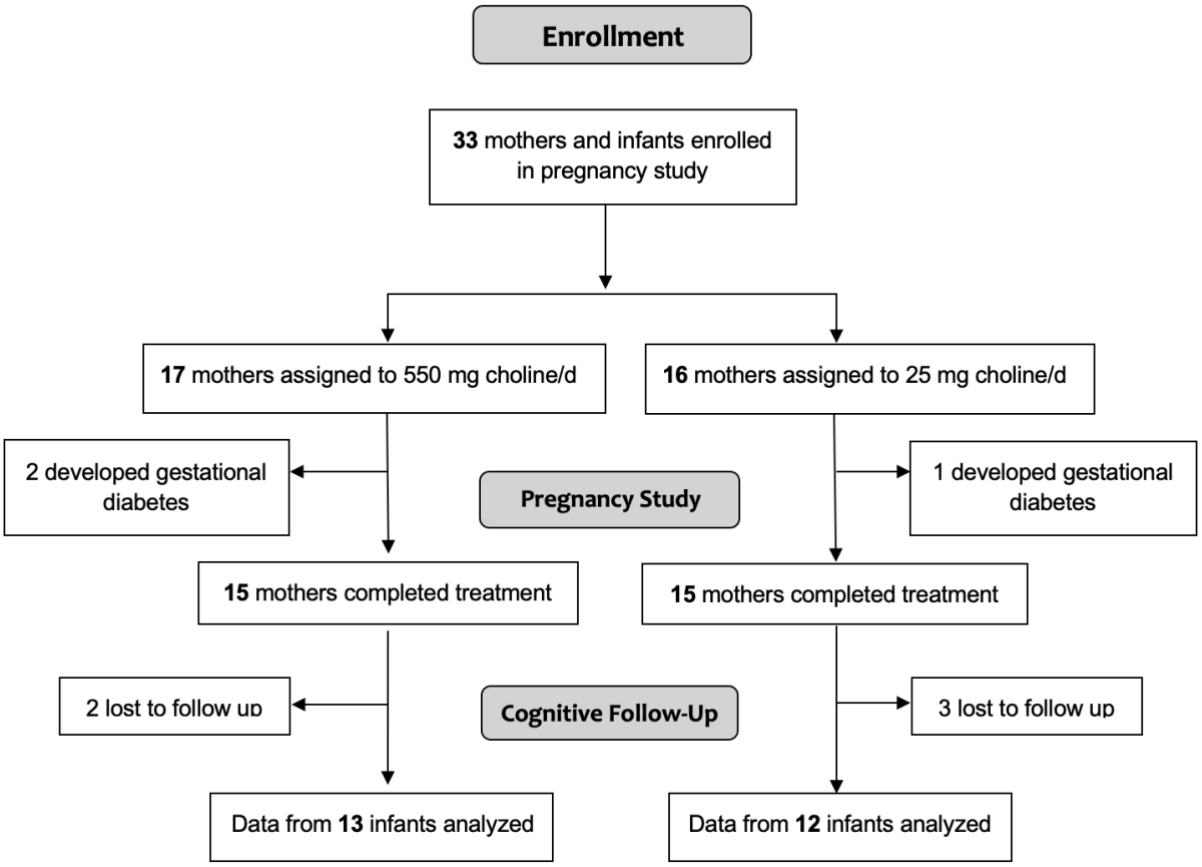

Figure 3. Trial profile. Flow of study participants through enrollment, randomization, and analysis. Due to the ongoing nature of the cognitive follow-up study, the writer remains blinded to individual participant treatment group assignments. Therefore, details regarding the cause of loss to follow-up for participants who completed the pregnancy study are not disclosed. This information will be available in the published manuscript of this study at a later date.

Data and Measures:

Once enrolled in the pregnancy study, participants were randomized to receive either 550 mg/d of supplemental choline chloride or control (25 mg/d deuterium-labeled choline tracer). These doses were chosen to approximate the higher choline intake level found to improve infant processing speed in Caudill et al.'s controlled feeding trial (480 mg/d and 930 mg/d) when taken alongside a woman's regular diet. Supplementation commenced at the initial study visit during the 12th-16th week of pregnancy and continued until delivery. At this visit, all participants were also instructed to terminate all of their routine dietary supplements and replace them with their assigned choline dose and study-supplied docosahexaenoic acid (DHA) and prenatal vitamins. Participants returned for additional study visits at 20-24 weeks and 28-32 weeks pregnancy to receive their daily choline supplements. All participants also consumed a once-daily prenatal vitamin/mineral supplement (Nature Made Prenatal Tablet; Pharmavite LLC; CA, USA) and a 200 mg/d DHA supplement (Nature's Way EfaGold Neuromins 200 mg DHA (plant source); DSM Nutritional Products; Netherlands). To monitor adherence, participants were instructed at each visit to return any unconsumed supplements at the following study visits.

During screening, information about maternal race, ethnicity, BMI, prior pregnancy complications, drug and alcohol use, supplementation use, education, and employment status were collected. This information was used to verify that treatment groups were balanced for variables potentially associated with cognitive outcomes. Food frequency questionnaires and Automated Self-Administered 24-Hour Dietary Recalls (ASA24) were administered at the first study visit to assess baseline choline and DHA intake. The ASA24 was also administered at six additional time points throughout the study to assess dietary intake of choline and nutrients that interact with choline metabolism, including DHA, eicosapentaenoic acid (EPA), folic acid,

vitamin B12, and vitamin B6. Participant adherence to the assigned level of choline intake was assessed based on choline metabolite concentrations in fasting blood samples and unconsumed supplement bottles collected at each of the three study visits. Following delivery, study staff who were blinded to treatment group assignment obtained information about due date, delivery date, gestational age, method of delivery, complications during the third trimester and delivery, birth weight, and gender from medical charts. These maternal and infant characteristics were collected for use in sensitivity analyses and assessment of potential bias due to loss to follow-up.

Behavioral Assessment -

To test the hypothesis that infants from women consuming a higher dose of choline will show faster information processing speed, as compared to infants from control mothers, infants were administered the Visual Expectation Paradigm (VExP) by one of two trained testers blinded to treatment group assignment. In addition to the VExP, the single-day two-hour testing protocol also included cognitive tasks measuring affect regulation, focused attention, and recognition memory. Results from these tasks will be reported at a later date. Throughout the 3-to 5-minute VExP task, the infant sat on a parent's lap in a darkened room while viewing a sequence of animation images appearing on the left or right half of a display screen (see **Figure 4**). Once the stimulus disappeared, the screen remained blank for one second (interstimulus interval- ISI) before the subsequent animation appeared for 700 ms. The latency difference between the onset of each stimulus and the infant initiating a visual fixation shift to the stimulus was measured over 31 trials. As shown in **Figure 5**, the first ten images appeared in an unpredictable left-right (L-R) sequence and were used to provide a baseline reaction time under conditions of spatial unpredictability. The subsequent 21 images followed a predictable L-R appearance pattern, termed the postbaseline phase. The VExP task measures latencies of saccadic eye movements

toward visual stimuli[8,80–82]. In developmental terms, information processing speed is a dimension of attention measured by reaction time (RT). Expectancy formation is measured by the proportion of anticipatory eye movements—fixation shifts to stimulus locations that occur before the stimulus has appeared on the screen, or so soon after the stimulus appears that the shift could not have been visually guided.

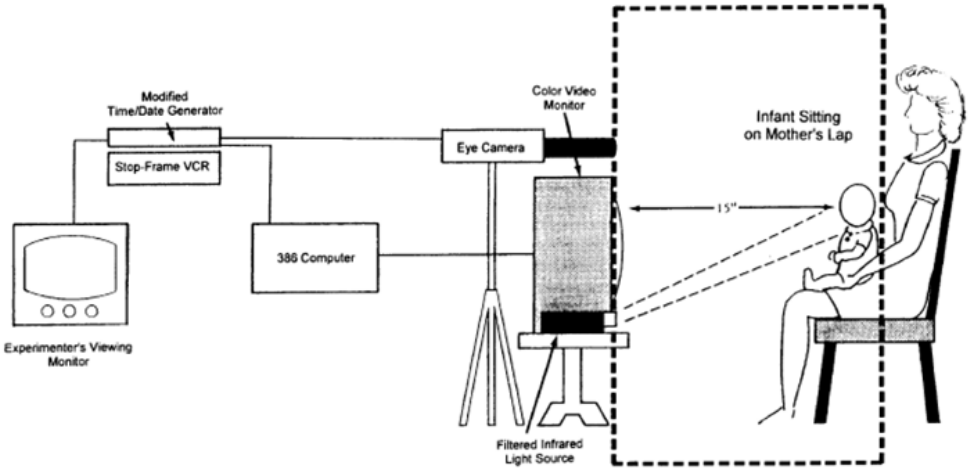

Figure 4. Illustration of Visual Expectation Paradigm task (VExP).

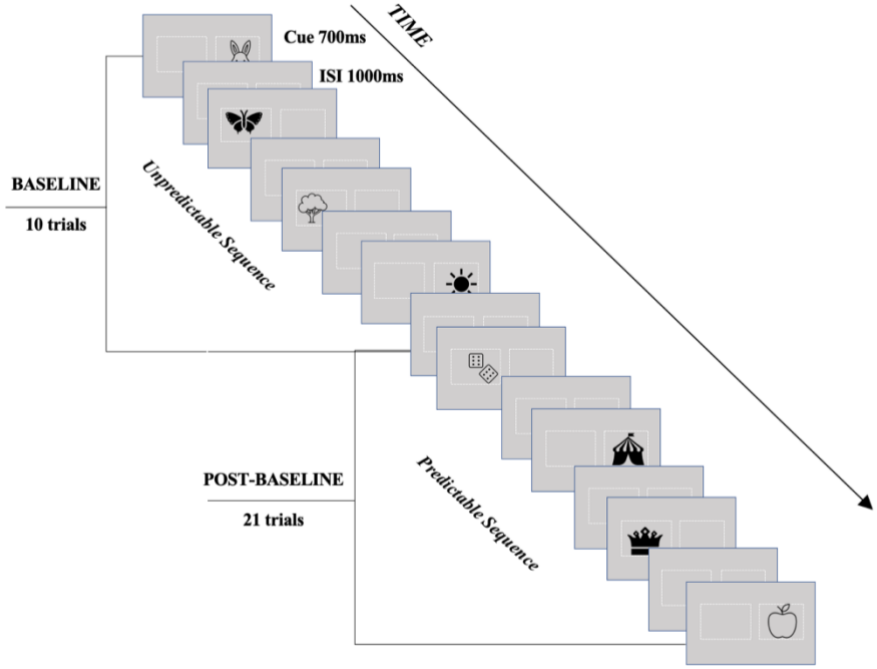

Figure 5. Illustration of VExP stimuli sequence. The task begins with a baseline trial of 10 stimuli presented in an unpredictable sequence. In the postbaseline phase, 21 stimuli are presented in a predictable sequence. Each stimulus appears for 700ms followed by a one-second interstimulus interval (ISI).

Ethics

Ethical approval for the present study was obtained from the Institutional Review Board for Human Participants at Cornell University in Ithaca, NY (USA). Written parental consent was obtained from all study participants.

Analytic Approach:

Coding:

Throughout the task, infrared corneal reflection videography was used to record the infant's eye region at a rate of 30 frames per second. These recordings were scored offline by a team of two raters blinded to the treatment group, including a graduate student and co-PI. The graduate student underwent extensive training using a validated, pre-existing coding system modified for the available technology. Following training, this student independently rated a total of 2604 events from the 84 VExP recordings. The co-PI performed criterion checks on 1209 events from 39 (46%) recordings identified as particularly challenging. Among these, the co-PI identified 71 events for reconsideration. Therefore, discrepancies occurred for a total of 5% of events reviewed by the co-PI and 2.4% of all possible events. Coding discrepancies were jointly resolved after the coding team reviewed, analyzed, and discussed the recordings. Codes for 59 events (<5% of events reviewed) were changed following review.

Dependent Variables:

To extract key endpoints, the rater assessed eye position relative to stimulus location for each video frame using Adobe Premiere Pro software (Adobe Premiere Pro, 2020). For each stimulus presentation, the infant could either react to the stimuli, anticipate its appearance, or engage in off-task behavior. Off-task behaviors included fussing, looking off-screen, or engaging with their caregiver in a way that interfered with their ability to orient to the animation. Saccade

latencies, defined as the delay between the stimulus appearance and the infant's initial eye shift toward the stimulus, were calculated for each stimulus appearance. Each saccade was then categorized as either reactive or anticipatory based on the latency value. Reactive saccades, occurring when visual information prompts rotation of the foveal region toward the stimulus, are defined as shifts occurring more than 133 ms after stimulus onset, which is the minimum required time for infants throughout this age range to process and react to visual information[83]. The latencies at which these ocular reactions occur provide a measure of reaction time (RT), which is an estimate of how quickly the infant registers the presentation of a peripheral visual stimulus, plans a saccadic eye movement, and initiates a motor response. The average RT over a series of trials is one measure of information processing speed. Longitudinal studies indicate that infant saccade RT is a reliable index of individual differences and age-related changes during infancy[80,83], predicts information processing speed and intelligent quotient scores in preadolescence [74–76], and executive functioning in later childhood[84]

The second form of saccade, anticipatory saccades (ANT) are defined as fixation shifts toward the upcoming stimulus location occurring less than 133 ms after stimulus onset or before the stimulus appearance[83]. The nature of this definition requires that ANT be motivated by expectations based on prior experience and information and is therefore thought to measure memory and attention. According to findings from Canfield et al., these expectancy-guided saccades do not show stability in individual differences over time and are only modestly reliable[83]. However, research from Benson et al. demonstrates a correlation between infant anticipations and early childhood IQ scores[74,76,85].

Our primary outcome of interest was mean saccade RT for the visually-guided fixation shifts. Each child contributed two data points for this variable, one baseline RT averaged across

the unpredictable L-R sequence images and one post-baseline RT averaged across the predictable L-R sequence images. Our secondary outcome was the percentage of anticipatory saccades. Again, each child contributed two data points for this variable, an anticipation percentage of visual saccades occurring during each L-R sequence presentation. Both outcomes of interest were measured at ages 5, 7, 10, and 13 months.

Statistical Analysis:

Power calculations indicated a sample size of 16 per treatment group to detect a significant effect of choline intake on biomarkers at $\alpha < 0.05$ and $\beta = 0.2$. Thirty-three participants were enrolled in the pregnancy study and 26 of these participants were successfully re-enrolled for the ancillary cognitive follow-up study, which employed a repeated measures design with assessments at four age time points. To assess whether our treatment randomization scheme produced groups with similar baseline characteristics, we compared maternal and infant characteristics using Student's t-tests for continuous variables and Fischer's exact test for categorical variables. Due to the ongoing nature of the cognitive follow-up study, the writer remains blinded to individual participant treatment group assignment and, therefore, comparison of baseline characteristics between participants lost to follow-up and those included in the final analyses have not been analyzed. This information will be available in the published manuscript of this study at a later date. SAS 9.4 Software (SAS Institute, Cary, NC, USA) was used to conduct statistical analyses, including linear and logistic mixed-model methods. All tests were 2-tailed and statistical significance was set at $P<0.05$ for main effects and $P< 0.10$ for interactions.

Primary Endpoint Analysis: Maternal Choline Intake and Mean Saccade RT

We first used an unadjusted model to explore whether the effects of MCS on offspring mean saccade RT demonstrated in the controlled feeding study could be replicated when women

consumed their usual diet. The unadjusted model represents the intention-to-treat analysis and avoids the possible complications of multicollinearity in an adjusted model. Because previous research has shown that saccade RT declines across age in infancy in an approximately exponential manner, age was entered into the model as the natural logarithm of testing age in days[83]. We also included fixed effects for the structural variables of treatment and stimulus sequence type. Random effects for the intercept and slope of age, animation sequence, and individual child were estimated using this model. Consistent with our objective to replicate findings from the controlled feeding study among women consuming an ad libitum diet, our *a priori* adjusted analyses included the same set of covariates as in the Caudill et al. study (maternal age, race, education, labor and delivery complications, and infant weight and gestational age) and all were entered as fixed effects. Like the unadjusted model, the adjusted model also included random effects for the intercept and slope of age, animation sequence, and individual child.

Secondary Endpoint Analysis: Maternal Choline Intake and Anticipation Percent

We used a logistic mixed-model regression method to examine our secondary outcome of interest, the percent of anticipatory saccades. Because research does not indicate that anticipatory saccades follow a nonlinear pattern of age-related change, age in days was entered as an untransformed variable in this model. The same fixed and random effects were entered into the model as those used in the RT unadjusted model. Again, our *a priori* analyses for anticipation percent adjusted for identical covariates of maternal age, race, education, labor and delivery complications, and infant weight and gestational age

RESULTS

Of the 33 mothers enrolled in the pregnancy trial, 91% (n=30) were eligible to participate in the cognitive follow-up study. Two mothers from the 550 mg choline group and one from the 25 mg choline group developed gestational diabetes and were subsequently excluded from the follow-up study. Twenty-six of the 30 eligible mother-infant dyads were re-recruited and enrolled into the postnatal cognitive study, 13 had received 550 mg/d choline supplementation, and 13 had received 25 mg/d of choline supplementation during pregnancy (see **Figure 1** for flow of participants through study-phases). One mother-infant pair was lost to follow-up after the 5-month study visit, and this infant became distressed during testing and did not complete the VExP task. Therefore, data from this child were not available for analysis.

Baseline demographic and clinical characteristics of mothers and infants included in the analysis are shown in **Table 1** and described below.

Characteristic	Overall (N=25)	25mg/d (N=12)	550mg/d (N=13)
Health and Demographics			
Age (years)	32.4(3.89)	31.92 (4.46)	32.77(3.59)
Body-mass index	23.51 (3.08)	24.65 (3.71)	22.45 (2.15)
Primiparous	10 (40%)	5 (41.67%)	5 (38.46%)
Education			
High School	2 (8.00%)	1 (8.33%)	1 (7.69%)
Bachelor's degree	5 (20%)	3 (25%%)	2(15.38%)
Master's degree	14(56%)	8 (66.67%)	6 (46.15%)
Doctorate/Professional	4(16%)	0	4 (100%)
Race			
White	22 (88%)	12 (100%)	10 (76.92%)
Black	1 (4%)	0	1 (7.69%)
Asian	2 (8%)	0	2 (15.38%)
Ethnicity			
Non-Hispanic	24 (96%)	12 (100%)	12 (92.31%)
Other	1 (4%)	0	1 (7.69%)
Pregnancy and delivery			
Gestation length (days)	279.8 (9.4)	278.3 (7.36)	281.2 (11.47)
Pregnancy/Labor complications	10 (40%)	5 (41.7%)	5 (38.5%)
Delivery method (vaginal)	21 (84%)	9 (75%)	12 (92.31%)
Birth outcomes			
Gender (female)	18 (72%)	10 (83.33%)	8 (61.54%)
Length (in)	19.41(1.1)	19.44(1.03)	19.38(1.24)
Weight (kg)	3.35(0.39)	3.38(0.35)	3.33(0.45)

Table 1. Maternal and infant health and demographics.
Numerical variables: means (SD). Categorical variables: counts (%). *P*> 0.08 for all variables.

Adherence

Choline metabolite concentrations were significantly higher in the choline versus control group, confirming high participant compliance to the assigned supplementation levels. Data illustrating this have been published elsewhere[79]. We estimated missed supplement doses based on the difference between the number of supplements administered at the previous visit and the quantity of unconsumed-supplement containers returned, accounting for the number of days since the last visit. High adherence was also supported by a low estimate of missed supplement doses between visits for all participants. Notably, calculations revealed that eleven participants achieved 100% adherence. Among the 15 participants that missed any dose, the average number of missed doses was 2.3 (range= 1-7 doses) over the entire study period, indicating that participants likely consumed their assigned supplement doses. No adverse effects of either choline dose were reported[79].

ASA24 Dietary Intakes:

Analysis of the six between-visit ASA24 dietary recalls revealed mean dietary intakes of DHA, EPA, folic acid, vitamin B12, and vitamin B6 were similar between treatment groups. Notably, throughout the study period, the mean intake of dietary choline was higher among the control group (mean=402 mg/d, SD=119.5 mg/d) as compared to the choline-supplement group (mean=337.2, SD= 65.71)—a non-significant difference (p=0.08). This finding is notable for two reasons. First, in combination with the assigned choline supplementation levels, these intakes contribute to an estimated mean total daily choline intake of 887 mg/d for the experimental group and 427 mg/d for the control group. These intake values approximate the supplementation levels of 930 mg/d and 480 mg/d from the previous controlled feeding study. Secondly, these diet-only intake levels are below the AI level, corroborating previous evidence indicating

widespread choline insufficiency among this population[21].

Due to the in-person research pause during the COVID-19 pandemic, we could not conduct assessments for seven infants at the 7-month time point, two at the 10-month time point, and one at the 13-month time point. Consequently, a total of 10 individual infant assessments were missed as a result of the COVID-19 pandemic. After research was reactivated, data for eight 13-month time points were collected when the child was older than 13 months (mean= 456 days, range= 409-501 days). An additional three individual infant assessments could not be conducted as one mother-infant pair relocated after the 7-month visit and another pair relocated after the 10-month visit. Technical problems caused poor video quality and prevented coding for two individual VExP recordings. Thus, the analytic sample includes data from 84 individual assessments for 25 mother-infant dyads, each tested at up to four different time points. Data were missing for a total of 16 individual infant assessments, as shown in **Table 2**.

# Participants Completing Testing n (%)		
Testing Age	**Choline n=13**	**Control n=12**
5-Month	11 (84.6)	11 (91.7)
7-Month	9 (69.2)	10 (83.3)
10-Month	11(84.6)	10 (83.3)
13-Month	12 (92.3)	10 (83.3)
Total:	43 (82.7)	41 (85.4)

Table 2. Completeness of VExP data at the different testing time points. Each cell indicates the total number of infant assessments completed at the respective testing age. The percentage of total possible assessments completed at each testing age is shown in parentheses.

Participant Characteristics

The baseline characteristics of our sample were predominantly similar between treatment groups, as shown in **Table 1**. Among mothers in the analytic sample, the large majority (n=22) were non-Hispanic White, one was mixed-ethnicity Black, and 2 were non-Hispanic Asian. Our

maternal participants were, on average, 32.4 years old and had a BMI of 24 kg/m^2. For 40% of our participants, this was their first pregnancy. 92% of our participants had completed at least a Bachelor's degree, and 78% of those had a Master's or Ph.D. Ten mothers reported pregnancy or labor complications, although only two infants in our analytic sample had health complications following delivery. These included small for gestational age and hyperbilirubinemia. Regarding delivery, 16% of participants had C-sections, and 84% had vaginal deliveries. The mean gestational age was 280 days, and 72% of the infants were female. On average, the infants were 49.3 cm long and 35 kg at birth. Analytical comparisons of baseline characteristics between groups were not statistically significant (all values $P>0.08$).

		Least Squares Mean				**Difference of Least Square Means**			
		25 mg/d Choline		**550 mg/d Choline**		**25 v. 550 mg/d Choline**			
Model	**Sequence**	**β**	**S.E.**	**β**	**S.E.**	**β**	**S.E.**	***P***	**CI (95%)**
RT									
	Baseline	365.77	12.56	348.85	12.24	16.91	17.53	0.338	(-18.09 - 51.91)
	Post-baseline	346.18	8.70	318.53	8.71	27.66	12.31	0.028	(3.09 - 52.23)
%ANT									
	Baseline	0.214	0.0440	0.224	0.0430	-0.01	0.06	0.867	(-0.13 - 0.11)
	Post-baseline	0.283	0.0340	0.303	0.0336	-0.02	0.05	0.687	(-0.11 - 0.08)

Table 3. Least Squares Means and Differences of Least Squares Means Results for Reaction Time and Percent Anticipations Unadjusted Models (n=25) *P<0.05, ** P<0.01, ***P<0.001

Model	**Reaction Time**		**Percent Anticipation**	
Effect	**F value**	**Pr>F**	**F value**	**Pr>F**
Treatment	4.11	0.047	0.68	0.412
Age	52.94	<0.0001	6.58	0.014
Sequence	5.35	0.025	1.34	0.254
Sequence x Treatment	0.27	0.607	0.44	0.507
Age x Sequence	0.56	0.458	0.12	0.735
Age x Treatment	0.20	0.659	0.44	0.508
Age x Sequence x Treatment	0.57	0.451	0.38	0.538

Table 4. Two-Way Tests of Fixed Effects for Reaction Time and Percent Anticipation Unadjusted Models (n=25) *P<0.05, ** P<0.01, ***P<0.001

Key Findings:

Mean Saccade RT:

RT data were available for 84 of 100 (84%) possible assessments. The unadjusted regression analysis estimated an overall mean saccade reaction time of 333.69 ms for infants in the 550 mg/d group, as compared to 355.98 ms for the control group. Indicating that infants from the higher choline group had RTs that were 22.3 ms faster than those from the control group (*P*= 0.047). Unadjusted least squares means and differences of least square means results for RT and percent anticipation variables are provided in **Table 3.** Model effects for the RT and percent anticipation variables are provided in **Table 4.** Our unadjusted model also exposed a significant effect of infant age in days at testing such that older children had faster RTs. This effect was consistent across treatment groups as we found no significant interaction effect between treatment and age (interaction *P*= 0.659). This suggests that the effect of choline on infant RT is consistent throughout this age range. As expected, our model revealed a significant effect of sequence type such that infants showed faster RTs while viewing the second, predictable animation sequence compared to the first, unpredictable sequence. Again, this effect did not differ by choline intake group (interaction *P*= 0.607).

The adjusted model included the set of covariates selected *a priori*; namely, infant age at testing, gestational age, birth weight, maternal race, age, and education and presence of labor and delivery complications. These covariates were chosen to replicate the model used in the prior controlled feeding study and the results essentially replicated the findings from the unadjusted model, revealing a difference in mean RT between the intervention groups of 25.4 ms (*P*= 0.042). Again, our adjusted model exposed significant effects of age (*P*= <0.0001) and sequence (*P*= 0.022) but none of the interaction effects between these structural variables were significant

(all values P>0.560). **Figure 6** illustrates the results of the adjusted model, showing infant mean saccade RT across the first year of life as a function of maternal choline intake level (550 mg/d v. control). Notably, adjustment for covariates identified *a priori* did not substantially alter the main effect of treatment, indicating that our findings are not model-dependent. However, in the adjusted model, the effect of maternal age at conception was significant (P=0.024) such that older maternal age, within the 24-to-39-year-old range seen in our sample, was associated with slightly faster RT.

Anticipation Percent:

In our unadjusted model, we found no significant effect of maternal choline intake on the percentage of infant predictive saccades (mean difference= 1.48%; P= 0.412). As expected, this model revealed a significant effect of infant age on the number of predictive saccades produced such that older infants had higher anticipation percentages. There was no effect of animation sequence type and no interactions between treatment, sequence, or age (all values P> 0.510).

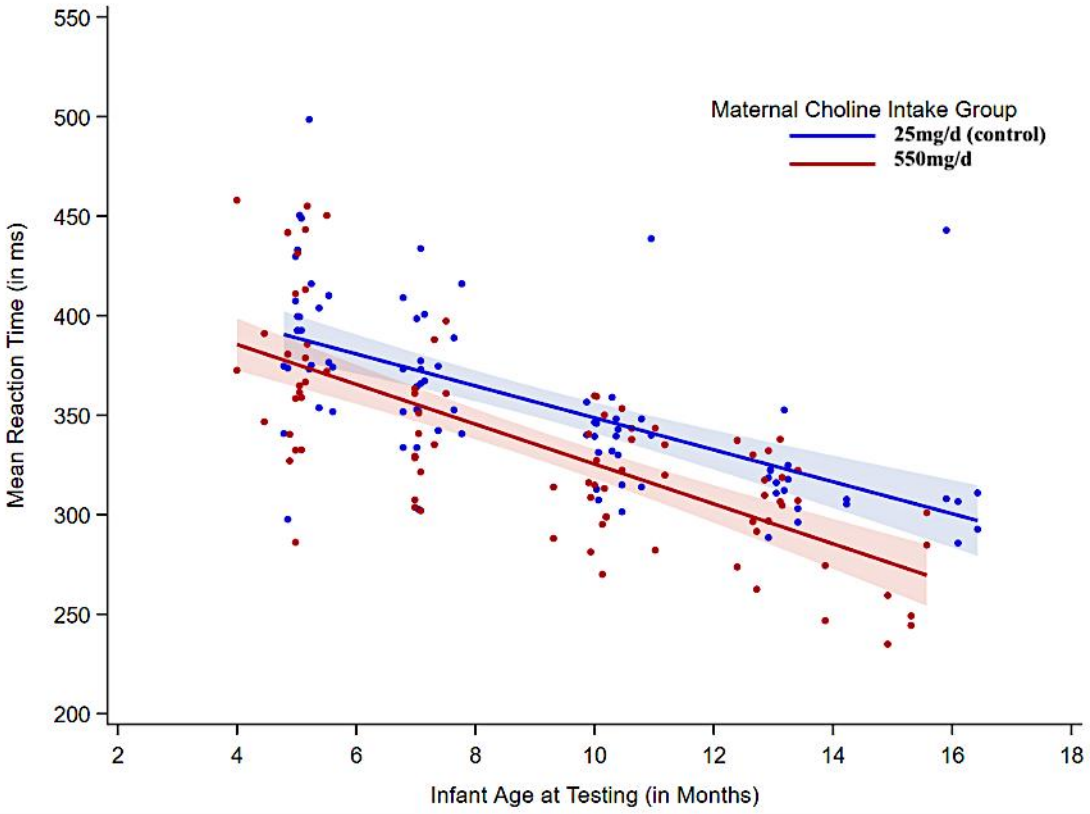

Figure 6. Illustration of the mean RT (in ms) as a function of second-trimester maternal choline intake level (550 mg/d v. control) and infant age at testing (in months), adjusted for infant age at testing, gestational age, birth weight, maternal race, age, and education and presence of labor and delivery complications. Each data point represents a predicted reaction time value calculated using our adjusted model. Lines depict least squares means estimates and shadings depict a 95% confidence band across age. The maternal choline intake effect is significant at (P= 0.042)

DISCUSSION

Several lines of evidence indicate that maternal choline supplementation in rodents has lasting beneficial effects on offspring cognitive functions. Although the previous controlled feeding study conducted in our lab demonstrated the efficacy of MCS on infant cognitive outcomes, the study lacked the real-world conditions necessary to assess the intervention's effectiveness. The few studies that have evaluated the effectiveness of MCS on functional outcomes in humans have yielded mixed and inconclusive results. Moreover, in these previous studies, it is unclear whether the cognitive tasks and measures were appropriate and sufficiently

sensitive to detect the effects of the intervention. Addressing these critical gaps, the presented study was designed to assess the effectiveness of MCS for improving offspring cognition using an outcome measure demonstrated to be sensitive to the benefits of MCS in the prior controlled feeding study.

The findings from this study demonstrate that daily prenatal supplementation of 550 mg choline chloride, alongside a woman's usual diet, improves infant processing speed, an early index of childhood cognitive abilities. Infants of mothers receiving 550 mg/d of choline chloride supplementation, initiated in the second trimester, had significantly faster saccade RTs when viewing unpredictable and predictable sequences of animations, than infants from the control group (**Figure 6**). These findings replicate results from Caudill et al.'s feeding trial in which third-trimester choline intake of double the AI improved infant reaction time. Perhaps more importantly, these findings also confirm the effectiveness of supplementing a mother's usual diet with choline to improve offspring cognition, a finding critical for the development of public health recommendations. Notably, despite substantially different study designs, the total choline intakes of the two groups in the present study are approximately equivalent to those in the prior controlled feeding study (480 mg/d v. 930 mg/d). And, remarkably, the mean difference in RTs between the higher and lower choline intake groups replicates those previously found. Taken together, these findings indicate that MCS at levels above the AI (450 mg/d) has beneficial cognitive effects on the offspring independent of a mother's background diet pattern, providing further support for the effectiveness of MCS as a public health strategy.

Recent findings from a 7-year follow-up of the children born to women in the prior controlled feeding study performed in our lab suggest that the cognitive benefits of increased maternal choline intake during pregnancy last well beyond the first year of life, extending at least

into childhood. When tested at 7 years of age, the children born to women in the higher choline intake group (930 mg/d) exhibited improved sustained attention, working memory, and problem-solving proficiency, relative to children born to women consuming 480 mg/d[9,10]. In light of this evidence, it is likely that consuming a choline supplement during pregnancy will not only improve infant processing speed during the first year of life but will also improve attention and memory during the school-age years. Rodent studies of MCS suggest that the long-term cognitive benefits of increased prenatal choline availability may be due to enduring structural differences in the central nervous system. One possibility is that MCS causes increased production of cell membrane and neuronal sheath constituents and this facilitates faster reaction times due to more efficient nerve signal transmission[5]. Moreover, choline donates methyl groups necessary for epigenetic modifications of hippocampal and cerebral genes, two brain areas integral to memory, attention, and learning[14,86,87].

Seemingly small increases in information processing speed during infancy may have lifelong significance. Longitudinal research by Rose et al. demonstrates that infant information processing speed measured using the VExP task predicts childhood executive functions (EF) at age 11, based on neuropsychological tasks of working memory, inhibitory control, and attention shifting. Similarly, 5-month-olds deemed to be more efficient information processors, based on look duration, exhibited superior performance on a battery of age-appropriate tasks tapping various EFs at 24, 36, and 48 months of age and across four different EF tasks again at 3 years of age, even after controlling for verbal intelligence[88]. Furthermore, increases in information processing speed during childhood account for age-related improvements in working memory[89]. These early-emerging and persisting executive functions are paramount to an individual's ability to problem solve, plan, reason, and self-regulate. Such capacities contribute not only to academic

success but also to lifelong wellbeing, as supported by evidence that executive functions, particularly inhibition, at ages 3 and 11 are predictive of physical health, personal finances, criminal behavior, and overall happiness in adulthood even after controlling for IQ, social class, and childhood home environment[90]. Collectively, these findings imply that early basic information processing abilities lay the groundwork for higher-level mental operations in preadolescence, which in turn predict adulthood outcomes. Accordingly, maternal choline supplementation is most beneficial for the offspring when delivered during the sensitive prenatal period.

Extending evidence from Caudill et al.'s controlled feeding study[8] and Ross et al.'s supplementation trial[71], these data provide important new evidence that daily prenatal choline supplementation alongside a woman's regular diet may be a practical and effective solution to address the high prevalence of choline inadequacy seen among pregnant women. Because choline inadequacy is ubiquitous and the risk of toxicity is vanishingly small, public health efforts to promote population-wide increases in prenatal choline intake are needed. In practice, these findings should be used by obstetricians, gynecologists, primary care physicians, and Registered Dietitian Nutritionists (RDN) to encourage choline-adequate dietary patterns and support routine prescription of supplemental choline beyond the quantities found in generic prenatal multivitamins and minerals. This message is consistent with recent recommendations from an expert panel consensus suggesting choline supplementation for all pregnant and lactating women[91].

Strength of Evidence:

This study has several strengths. First, the randomized, placebo-controlled design of the study allows for drawing strong causal inferences. Moreover, the evidence for high supplement

adherence and ASA24 dietary intake data indicate that total choline intake was substantially different between the treatment groups. An additional strength of our study was that the mean total choline intake levels of our experimental groups, derived from participant's regular diet and their assigned supplementation level, approximated the intake levels prescribed in the previous controlled feeding study[8]. Replication of participant choline intake levels from the previous study allowed us to compare the effects of MCS on information processing speed between choline delivered through a strict diet and choline supplemented to a women's typical diet. Had the choline intake levels of these studies differed, variation in choline doses may have, in part, accounted for differences in information processing speed, precluding the direct comparison of outcomes between studies.

An additional strength of our study was that all participants, researchers, and study staff remained blinded throughout data collection, assessment, and analysis. Notably, treatment group assignments were not disclosed until all VExP recordings had been coded and all statistical analyses were completed, preventing analytical bias. Another strength of this study was the repeated measures design which allowed us to measure the effect of maternal choline intake on offspring neurobehavioral functioning at four timepoints, improving the validity of our findings. The longitudinal nature of this study also allowed us to assess the effects of MCS on information processing speed throughout the first year of postnatal life, providing evidence for the enduring benefits of supplementation.

An additional strength of this study was the use of the VExP task as the primary outcome measure to assess the effects of MCS on infant cognitive functioning [74,80]. As noted above, this measure had proven sensitive to the effects of MCS in our prior choline feeding study. In addition, the VExP consistently provides an objective, quantitative assessment of information

processing speed, and is one of the few infant measures that has been shown to predict IQ in adolescence[78]. "A final strength of the study is that we used choline chloride as the supplemental form of choline; this form of choline has a higher bioavailability than the form (phosphatidylcholine) used in the two prior human choline supplementation trials[71,72].

This study also has important limitations. First, our small sample size raises a concern that the findings, albeit statistically significant, might not reflect a true effect of the different levels of maternal choline intake. It should be noted, however, that despite a small sample and considerable loss of data due to the COVID-19 research shutdown, our study was adequately powered to detect a significant effect of MCS, with an effect size that was identical in magnitude to the effect seen in the controlled feeding study. Moreover, although the temporary research shutdown resulted in a wide variation of infant ages at the 13-month testing time point, we could account for this variation in our statistical analyses. Therefore, the results of this study were unchanged despite the many challenges of COVID-19.

A second limitation of the study is that the lack of diversity in our sample limits the generalizability of our findings beyond the largely non-Hispanic White, highly educated population of our study. Similarly, our sample may only represent women who are willing to adherence to a strict supplementation routine, attend frequent pre-and postnatal lab appointments and undergo specimen collection procedures. It is plausible that the high adherence and commitment of our participants are not representative of the average woman of childbearing age and, therefore, the magnitude of MCS effects may not be as substantial for the general population of pregnant women.

A final limitation of our study is that, although the VExP task provides an objective, quantitative assessment of information processing speed, RT captures only a narrow dimension

of attention which is one of many cognitive capacities. Future research should employ multiple paradigms that tap various aspects of attention (sustained, alerting, focused, executive) as well as other cognitive domains and executive functions to provide more robust support for the role of choline in early differences of diverse intellectual abilities. Tasks with a higher degree of ecological validity, such as assessments of attention during spontaneous play with toys, could provide information about the possible role of MCS on the types of attentional behaviors infants engage in during daily activities.

Future Research:

The findings of our study provide encouraging support for the effectiveness of maternal choline supplementation as a public health strategy and lay the groundwork for future studies. However, our study was not designed to determine the optimal effective choline dose or delivery method or to elucidate the supplementation needs of high-risk mothers. These remaining questions must be answered before MCS can be implemented at the population level. Importantly, our study assessed the effectiveness of MCS delivered only in the second and third trimesters of pregnancy. As such, we cannot predict the effects of supplementation initiated earlier or later in pregnancy. Of note, choline supplementation commenced in the 3rd trimester during the controlled feeding study, raising the question of whether MCS alongside a woman's regular diet would yield the same neurodevelopmental benefits if initiated only in the third trimester.

Furthermore, given that nearly half of the population has gene polymorphisms affecting the metabolism of methyl-donor nutrients, including choline[92], forthcoming dietary recommendations will need to account for the elevated requirements of this subpopulation. Thus, additional clinical trials with substantially larger and more diverse samples will be critical to

confirm the benefits of increased maternal choline intake on child outcomes and to understand whether these benefits vary based on maternal genetic profiles or risk factors that increase dietary choline requirements. Future multi-site trials must also compare numerous choline doses initiated at various stages of pregnancy to fully evaluate the optimal effective choline dose. Finally, studies employing comprehensive assessments of offspring cognition and socioemotional functioning, beyond the simple VExP visual attention task, are needed to characterize the range of offspring functions that may benefit from MCS.

Distinguishing the consequences of choline insufficiency from those of deficiency remains challenging given the lack of dose-response studies and poorly grounded AI recommendations. Furthermore, a variety of common single-nucleotide polymorphisms (SNP) in genes that regulate choline substantially alters an individual's requirement for endogenous choline sources, suggesting that the AI may be even more erroneous for these individuals[93,94]. Accordingly, dose-response trials will be critical to explore differences in choline requirements based on genetic variations and life-stages to establish an RDA and EAR and further delineate recommendations for those at increased risk of inadequacy. Finally, to confirm the potential enduring benefits of MCS beyond the first year of life, future studies should plan for longer follow-up periods extending into toddlerhood and early adolescence.

CONCLUSION

The presented findings demonstrate that choline supplementation alongside a woman's normal diet improves infant information processing speed across the first year of life, confirming the beneficial effect of increased prenatal choline availability for optimal neurodevelopment, as seen in previous human and rodent studies. This is important because infant information processing speed is a predictor of adolescent IQ and this early-developing cognitive ability is

foundational to higher-order executive functions that predict later-life health and wellbeing. Notably, these results replicate findings from Caudill et al.'s feeding trial in which third-trimester choline intake of double the AI improved infant reaction time and, more importantly, extend the translatability of such evidence to a more practical and feasible supplementation method. In light of these findings, routine MCS beyond the insufficient quantities found in generic prenatal multivitamins and minerals may optimize the cognitive abilities of subsequent generations and, in turn, improve life-long brain outcomes.

IMPLICATIONS

The widespread inadequate choline intake of pregnant women in the US, though problematic, is unsurprising. Considering many pregnant women seek dietary advice from media, dietary agencies, and health practitioners, it is concerning that only 10% of health professionals endorse moderate familiarity with choline and a mere 6% of obstetricians and gynecologists report recommending choline-rich foods to their patients[95]. Moreover, the Dietary Guidelines for Americans (DGA) that clinicians, consumers, and producers rely heavily on to make evidence-based dietary choices provides conflicting suggestions. Contrary to previous advice to limit saturated fat and dietary cholesterol, the most recent iteration of these guidelines now recommends the consumption of eggs, meats, seafood, and legumes to achieve adequate choline intakes during pregnancy. Though this is progress, the long-standing undertone discouraging animal-product consumption and the new emphasis on plant-based diet patterns persist in public and medical dialogue. Consequently, an increasing number of women have adopted vegetarian and vegan diets in the past decade[96]. Despite mixed evidence regarding the effects of such diets on fetal health outcomes, findings that the mean intake of choline among

vegetarians is a mere 192 mg/d[97] confirm that choline-related public health messaging is failing pregnant women.

Evidence to date supports the addition of adequate choline levels to prenatal supplements to fill this nutritional gap. Considering that 1) the choline Tolerable Upper Limit (UL) for adults of 3500 mg/d is nearly 8x the AI level and was established based on rare case reports of hypotension and fishy body odor observed at this intake level[12], 2) no adverse events of choline supplementation at levels 2x the AI have been reported in our studies, and 3) multiple studies have demonstrated clear neurodevelopmental benefits for offspring of mothers consuming choline at levels above and beyond the AI, supplementation of 100% the AI in all prenatal vitamins is warranted. Remarkably, in 2017 the American Medical Association recommended that prenatal vitamin supplements contain 'evidence-based' quantities of choline to ensure proper brain and spinal cord development[16]. However, manufacturers have been slow to take action for two reasons. First, in the absence of RDA and EAR guidelines for choline, 'evidence-based' remains ambiguous. And, secondly, adding sufficient choline to prenatal supplements would significantly increase their physical size, potentially reducing consumer desirability to purchase such products.

Choline remains overlooked from a clinical standpoint as well. Observational studies have found fatty liver and liver damage in patients receiving total parenteral nutrition (TPN) and, despite trace quantities of choline in TPN lipid emulsions, 85% of patients receiving total parenteral nutrition had low plasma-free choline concentrations (TPN)[98]. In spite of recommendations from the American Society for Parenteral and Enteral Nutrition (ASPEN) for the routine addition of choline to adult and pediatric PN formulations, a commercially available parenteral product has not been developed[99]. Thus, for the TPN-treated critically ill infant or

toddler who would otherwise receive choline-rich breastmilk or formula, the neurodevelopmental consequences of choline deficiency may be detrimental.

Nutritional choline intake and prenatal choline supplementation are critical to healthy fetal brain development, particularly during the second and third trimesters. Studies have reported that maternal choline intake is inversely associated with risk of adverse pregnancy outcomes including neural tube defects[58,59], preterm delivery[60], and risk of disability and cognitive impairment in children exposed to alcohol[63,64], marijuana[61], or severe infections[62] in utero. Meanwhile, maternal choline supplementation above AI levels has now been shown to improve various aspects of offspring cognitive performance throughout childhood[8–10], and to mitigate against the adverse effects of prenatal alcohol exposure[64]. In light of growing evidence that the AI for choline may itself be inadequate to achieve optimal neurodevelopment and a vast majority of pregnant women fail to achieve this unfounded recommendation level, the need for larger dose-response randomized controlled trials to establish appropriate recommendations is urgent.

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