

THE EFFECT OF MATERNAL CHOLINE INTAKE ON CHILD ATTENTION
AND MEMORY: A SEVEN-YEAR FOLLOW-UP

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Background: Decades of rodent research have demonstrated that perinatal maternal choline intake is important for offspring cognition throughout the lifespan, most notably attention and spatial cognition. However, few human studies have evaluated the effect of maternal choline supplementation during pregnancy or lactation on child cognition, particularly during school-age. **Objective:** To assess the effect of maternal choline supplementation during pregnancy or during exclusive breastfeeding on child cognitive functioning at age 7 years. **Design:** Women in their 27th week of gestation (“pregnancy cohort”) or in the early post-partum period while exclusively breastfeeding (“lactation cohort”) were recruited to take part in controlled choline feeding studies. Within each cohort, women were randomized to consume either 480 mg choline/d (approximately the Adequate Intake [AI] for pregnancy) or 930 mg choline/d for 12 weeks (pregnancy cohort) or 10 weeks (lactation cohort). Ancillary follow-up studies were conducted to assess child attention, memory, and intelligence at age 7 y. **Results:** In the pregnancy cohort (n=20), children whose mothers consumed 930 mg choline/d during their 3rd trimester exhibited enhanced attentional control, including improved sustained attention, compared to children whose mothers consumed 480 mg choline/d. Furthermore, children whose mothers consumed 930 mg choline/d during their 3rd

trimester exhibited enhanced visuospatial short-term memory capacity compared to children whose mothers consumed 480 mg choline/d. In the lactation cohort (n=18), no consistent benefit of maternal choline supplementation was detected. However, a lack of group differences in breastmilk total choline concentrations in those re-recruited likely precluded a strong test of the hypothesized effects of postnatal maternal choline supplementation on child cognition. **Conclusions:** Maternal consumption of approximately double the choline AI during the 3rd trimester of pregnancy had beneficial effects on child attention and memory at age 7 y compared to approximately the AI. This study provides the first evidence from a randomized controlled trial that prenatal choline supplementation improves cognitive functioning on behavioral tasks. These preliminary, but compelling, data suggest that the choline AI for pregnancy may not be sufficient to promote optimal offspring cognition. Larger clinical trials are needed to confirm and extend findings from this small follow-up study.

BIOGRAPHICAL SKETCH

Dr. Charlotte L. Bahnfleth was born in Cincinnati, Ohio but spent most of her childhood in State College, Pennsylvania. She is the daughter of architectural engineering professor, Dr. William P. Bahnfleth, and church organist, Mary L. Bahnfleth. Charlotte received her Bachelor of Science with honors and high distinction in Nutritional Sciences and Psychology from the Schreyer Honors College of the Pennsylvania State University in 2013. Under the supervision of Dr. Laura E. Murray-Kolb, Charlotte's undergraduate honors thesis explored the effect of early childhood micronutrient supplementation on child behavior during the early school-age years in Nepal. Charlotte matriculated at Cornell University in the Fall of 2014 to begin her Ph.D. in Human Nutrition within the Division of Nutritional Sciences. Under the advisement of Dr. Barbara J. Strupp, Charlotte conducted a 7-year follow-up of a randomized, controlled maternal choline feeding trial to assess the long-term effects of choline intake during pregnancy or lactation on child cognition for her dissertation.

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CHAPTER 1: INTRODUCTION

1.1 Developmental Origins of Cognition

The “first 1000 days” of life, approximately conception through a child’s second birthday, coincide with the most active and rapid period of neurodevelopment¹. During this time period, the developing brain is particularly sensitive to biological and psychosocial factors such as stress and inflammation, social support, and diet². Nutrition during pregnancy and early postnatal life is a particularly important influence on brain development, and deficiencies in any of several key nutrients can result in long-term dysfunction for the child³. One such nutrient is choline, the focus of this dissertation. Choline is a tri-methylated quaternary ammonium salt which plays an important role in the structural integrity of biological membranes, the synthesis of the neurotransmitter acetylcholine, and the metabolism of methyl groups⁴.

1.2 Maternal Choline Intake: Recommendations and Practice

The Institute of Medicine (now National Academy of Medicine) established choline as an essential nutrient in 1998. Insufficient evidence existed to determine a recommended dietary allowance (RDA) for choline at that time. Instead, an adequate intake (AI) level was set, based on the amount of choline needed to prevent liver dysfunction in men, based on data from a single study⁵. No published research on the choline requirements of women was available at the time of the AI’s establishment. Instead, adult female choline AIs were extrapolated

from the adult male AI, with adjustments made based on reference weights and the demands of pregnancy and lactation, namely fetal growth and secretion of choline into human milk. Studies which evaluated the effect of maternal choline intake on outcomes related to fetal or early child development such as cognitive or behavioral functioning were not available. Choline intake recommendations for adults are presented in **Table 1.1**.

Table 1.1 Choline Adequate Intake (AI) for adults by life stage

	Choline AI (mg/d)
Males	550
Non-Pregnant, Non-Lactating Females	425
Pregnant Females	450
Lactating Females	550

Despite the presence of choline in commonly consumed foods, such as eggs, beef, and fish⁶, few North American women of reproductive age meet the choline AI. Research suggests that approximately 90% of pregnant and lactating women in North America consume less than the choline AI, with average intake ranging from approximately 300 mg to 380 mg choline/d⁷⁻¹¹. Deficiency cannot be inferred based on consumption below the AI, but emerging evidence from rodents and humans suggest that the choline AI for pregnant and lactating women may not meet physiological demands during these life stages, leading to sub-optimal fetal and child cognitive development.

Rodent research has found that pregnancy and lactation cause a

pronounced depletion of maternal choline pools despite the consumption of a standard chow presumed to provide adequate amounts of choline¹²⁻¹⁴. These findings of increased choline demands during pregnancy in rodents are consistent with the results of a randomized controlled feeding study in humans, which showed that pregnant women who consumed choline at 480 mg/d (approximately the AI) or 930 mg/d (approximately double the AI) for 12 weeks had significantly lower circulating concentrations of choline metabolites compared to non-pregnant women who consuming the same amount of choline¹⁵. Furthermore, pregnant women who consumed approximately double the choline AI had higher circulating concentrations of choline metabolites compared to pregnant women who consumed approximately the AI¹⁵. This higher choline intake level did not result in increased urinary excretion of choline, suggesting the capacity of cells to utilize choline was not exceeded¹⁵. Finally, over 25 years of research in rodents has demonstrated that increasing maternal choline intake during pregnancy and the early postnatal period produces life-long cognitive benefits in offspring, suggesting that higher maternal choline intake may be needed to fully meet fetal and infant demands.

1.3 Choline & Cognition: Evidence from Animals

Choline plays important roles in fetal development, underscoring the importance of adequate choline intake during pregnancy. First, choline-derived phospholipids, such as phosphatidylcholine and sphingomyelin, are integral components of biological membranes and are in high demand during the rapid cell

division, growth, and myelination associated with fetal development. Second, acetylcholine, a neurotransmitter for which choline is a precursor, influences many processes in the developing brain such as cell proliferation and differentiation, neurogenesis, gliogenesis, cell survival, morphology and migration, and synaptic plasticity^{16,17}. Finally, choline is the major source of methyl groups in the diet (as betaine), and the availability of choline during fetal development is known to alter promoter region DNA^{18–20}.

The clearest evidence that prenatal choline exposure is functionally important for the offspring comes from the decades of rodent research demonstrating that offspring spatial cognition is improved by prenatal choline supplementation^{21,22}. For example, several studies have found that offspring of choline supplemented dams commit fewer errors in a radial maze compared to offspring of dams maintained on a standard diet^{23,24}. Importantly, these improvements in spatial cognition have been shown to persist through adulthood and into old age, resulting in the offspring of choline supplemented dams experiencing substantial reductions in aging-related cognitive decline²¹. In contrast to the improvements seen with prenatal choline supplementation, prenatal choline deprivation impairs offspring spatial cognition^{21,22}.

A smaller body of evidence has shown that maternal choline intake during pregnancy affects the attentional functioning of their offspring. For example, mice born to choline supplemented dams exhibited superior performance on a 5-choice visual attention task^{25,26}. Another study in mice found that performance on a signal detection task varied by prenatal choline exposure, with mice born to

choline supplemented dams exhibiting superior performance and mice born to choline deficient dams exhibiting poorer performance compared to controls²⁷. Additionally, rats exposed to higher choline prenatally had an increased ability to divide attention between multiple stimuli²⁸. It is also possible that improved attention contributes to the observed benefits of maternal choline supplementation on spatial maze performance²⁹, the most common test of spatial cognition utilized in this area of research.

Although several rodent studies have assessed the effect of perinatal choline supplementation on offspring cognition, none have evaluated the effect of maternal choline supplementation during lactation alone on offspring cognition via breastmilk. Instead, studies of postnatal choline supplementation have provided the supplement directly to the offspring during the period of lactation and weaning^{23,30}. This research has revealed beneficial effects of postnatal choline supplementation on spatial cognition, but these benefits were smaller than those seen with prenatal choline supplementation^{23,30}. The more subtle effects of postnatal choline supplementation may be because the neurodevelopmental processes that occur during this period are less sensitive to choline exposure than those occurring prenatally. However, it is possible that the potential to benefit from supplemental choline during the early postnatal period is smaller because rat milk is already rich in choline^{31–33}.

In addition to the role early life choline exposure appears to play in typical neurodevelopment, prenatal and/or postnatal choline supplementation has also been shown to have neuroprotective effects in diverse conditions of cognitive

dysfunction. For example, prenatal choline supplementation alleviates a variety of cognitive-behavioral deficits in rodent models of Alzheimer's Disease³⁴, autism³⁵, Down syndrome^{25,26,36,37}, *in utero* stress exposure³⁸, prenatal iron deficiency³⁹, and prolonged epileptic seizures⁴⁰⁻⁴³. Furthermore, postnatal choline supplementation directly to the offspring during the period of lactation has been shown to improve offspring functioning following neural insults including fetal alcohol exposure⁴⁴⁻⁴⁸ and neonatal iron deficiency⁴⁹ in rodents.

Many studies have been conducted to elucidate the neural mechanisms that may underlie the observed improvements in offspring cognitive functioning following prenatal and/or postnatal choline supplementation. The findings of improved spatial cognition and memory are consistent with the body of research that demonstrates maternal choline supplementation improves the functioning of the offspring hippocampus^{50,51}, and cholinergic system, which has been shown to modulate memory function⁵². Specifically, maternal choline supplementation has been shown to produce a variety of beneficial structural^{23,36,37,53-56}, electrophysiological^{57,58}, and neurochemical^{30,56,59-63} changes to the septo-hippocampal system of offspring. In comparison, few studies have assessed the neural changes that may underlie observed improvements in offspring attention. However, it is plausible that changes to cholinergic neurons in the basal forebrain may mediate these effects. Past research has demonstrated the importance of basal forebrain cholinergic projections to the cortex in sustained, selective, and divided attention, such as observations of increased cortical acetylcholine release during sustained attention tasks⁶⁴⁻⁶⁷. Structural changes to the basal forebrain have

been detected in offspring following perinatal choline supplementation⁵⁴ and prenatal choline deprivation^{53,55}.

1.4 Choline & Cognition: Evidence from Humans

Despite the extensive body of animal research demonstrating the importance of maternal choline intake for offspring cognition, few observational studies or randomized controlled trials (RCTs) have examined the relationship between maternal choline intake or plasma/serum choline metabolite levels and child cognition. The results of these few studies have so far been inconclusive. A brief discussion of the available observational and experimental research follows.

Observational Research: Pregnancy

Most observational research on the importance of early-life choline exposure has focused on the prenatal period. First, in a prospective cohort study (n = 154) conducted in Vancouver, Canada, fasting maternal plasma free choline and betaine at 16 weeks gestation, but not at 36 weeks gestation, was positively associated with offspring performance on the cognitive skills domain of the Bayley Scales of Infant Development, Third Edition (BSID-III) at 18 months of age¹¹. Second, in a prospective cohort study (n = 404) conducted in Birmingham, Alabama, non-fasting maternal serum concentrations of free and total choline across the 2nd and 3rd trimester were not associated with child performance on the Wechsler Preschool and Primary Scale of Intelligence-Revised (WPPSI-R) at 5 years of age⁶⁸. Serum concentrations of free and total choline in cord blood also did not correlate with child performance on the WPPSI-R in this study⁶⁸.

In contrast to these studies which used maternal plasma/serum choline concentrations as their measure of prenatal choline exposure, a prospective cohort study conducted in eastern Massachusetts administered food frequency questionnaires (FFQs) to assess maternal choline intake during the 1st and 2nd trimesters of pregnancy^{9,69}. When children were assessed at 3 years of age in this cohort (n = 1210), no association between maternal choline intake and child performance on the Peabody Picture Vocabulary Test III (PPVT-III) or the Wide Range Assessment of Visual Motor Abilities (WRAVMA) was detected⁶⁹. In this same cohort at 7 years of age (n = 890), an assessment of visual memory, the Wide Range Assessment of Memory and Learning, Second Edition (WRAML2), Design and Picture Memory subtests, was administered in addition to an assessment of intelligence⁹. Second trimester choline intake was significantly, positively associated with performance on the WRAML2⁹. Specifically, children in the highest quartile for maternal choline intake scored 1.4 points higher on the WRAML2 compared to children in the lowest quartile⁹. A similar, but weaker and nonsignificant trend for 1st trimester choline intake was detected⁹. No statistically significant association between choline intake and child intelligence (measured by the Kaufman Brief Intelligence Test, Second Edition [KBIT-2]) was detected in this study⁹. However, the results for nonverbal KBIT-2 scores approached statistical significance, with the highest maternal choline intake quartile scoring 3.5 points higher than the lowest maternal choline intake quartile⁹.

One potential explanation for the mixed findings of these observational studies is the varied measures of choline exposure. Two studies used plasma or

serum choline metabolite levels as their measure of prenatal choline exposure^{11,68}. Although plasma free choline is a specific biomarker of choline status, it is under tight regulation and is relatively unresponsive to moderate changes in habitual choline intake⁷⁰. Consistent with this fact, maternal choline intake (assessed with an FFQ) was only weakly correlated ($r = 0.2$) with fasting plasma free choline for the one study it was available¹¹. Furthermore, in one study blood samples were collected in a non-fasting state, allowing for the possibility that samples were reflective of recent choline intake and not status⁶⁸. In contrast, prenatal choline exposure was assessed with a semi-quantitative FFQ in the cohort from eastern Massachusetts^{9,69}. FFQs can provide a relatively good measure of habitual dietary intake, but they are less precise than a 24-hour recall and are prone to measurement error⁷¹. It is unclear which measure may provide the best assessment of prenatal choline exposure, but in all cases there are concerns about exposure misclassification, either due to errors in estimating dietary choline through self-report, the use of non-fasting choline metabolite data, or the relative unresponsiveness of plasma choline to modulations in choline intake.

The mixed findings of these observational studies may also be a result of the measures of child cognitive performance selected. Of the two studies that detected positive associations between prenatal choline exposure and child cognition, one directly addressed visuospatial memory⁹. In no observational study was child attention assessed directly. Those studies that found no statistically significant associations between maternal choline intake or plasma/serum choline metabolite levels and child cognition used measures which assess cognitive

functioning more broadly, such as the WPPSI-R and the PPVT-III^{9,68,69}. Because rodent studies of maternal choline supplementation have consistently detected beneficial effects on offspring spatial memory^{21,22}, it is possible that tests of memory in humans may be more sensitive to the putative effects of maternal choline intake compared to standardized intelligence tests.

A final consideration of these observational studies is the range of maternal choline intakes present in the cohorts. Importantly, estimated choline intakes in the two cohorts for which they were available show that the majority of women ($\geq 74\%$) in these studies consumed choline at levels below the AI^{9,11}. Similar patterns of choline intake among pregnant women have been reported in other studies, with very few women consuming choline at levels above the AI^{7,72,73}. Therefore, this body of observational research cannot assess whether there are benefits of maternal choline supplementation at intakes greater than the current AI.

Observational Research: Lactation

Only one observational study has assessed the association between postnatal choline exposure and child cognition. Specifically, the relationship between free choline, lutein, and docosahexaenoic acid (DHA) levels in breastmilk and infant recognition memory, as assessed through electrophysiological measures, was investigated⁷⁴. In this study, breastmilk free choline concentrations at 3 to 5 months postpartum was associated with better recognition memory in 6-month-old exclusively breastfed infants, but only when levels of breastmilk lutein and DHA were also high ($n = 55$)⁷⁴. This study provides the only published data in humans regarding the potential importance of breastmilk choline levels for child

cognitive development, but the conclusions that can be drawn are limited. First, this study elected to only utilize free choline in their analyses⁷⁴. Other forms of choline including phosphocholine, glycerophosphocholine, and sphingomyelin are more abundant than free choline in breastmilk⁷⁵ and whether free choline concentrations are an accurate proxy for total choline concentrations in breastmilk is unclear. Second, only one sample of breastmilk was collected per woman. Therefore, any natural variation in breastmilk choline that occurs throughout the day would not have been captured by this study, leaving open the possibility that samples were not reflective of usual breastmilk free choline content.

Importantly, this study did not collect data on maternal choline intake during lactation or pregnancy. Because no information was available on postnatal choline intake, the extent to which maternal diet during lactation contributed to the choline content of breastmilk could not be evaluated⁷⁴. Additionally, it is not possible to evaluate potential associations between prenatal choline intake and breastmilk choline content. The nutrient content of breastmilk could be reflective of the maternal diet during pregnancy, due to similarities in diet across the periods of pregnancy and lactation. Therefore, there remains a possibility that the observed relationship between breastmilk choline concentration and child cognitive functioning could be explained in part by exposure to choline *in utero*.

Randomized Controlled Trials (RCTs)

Because causal inference is precluded in observational studies and the existing body of observational research is potentially affected by issues of exposure misclassification, outcome misclassification, and uncontrolled confounding,

evidence from RCTs on this topic are needed. However, only three published RCTs to date have evaluated the effects of maternal choline supplementation on child cognition or brain function. No RCTs have assessed the exclusive effect of maternal choline supplementation in the post-partum period during breastfeeding on child cognition. One of these RCTs randomized pregnant women to either supplemental choline (900 mg/d) or a placebo beginning in the 2nd trimester (n = 93); consumption of a choline supplement (15 mg/d) or placebo continued with the infants after the women gave birth⁷⁶. Infants in the supplement group displayed improved cerebral inhibition during an auditory evoked response task at 5 weeks of age compared to controls; this benefit of maternal choline supplementation appeared to be transient, however, as it was not seen when the children were 13 weeks of age⁷⁶. Cerebral inhibition is associated with a reduced risk of attentional disorders as the child ages and is therefore broadly consistent with the findings of improved attention with prenatal choline supplementation in rodents. However, no direct, behavioral measure of attention was included in this study. When a subset of these same children was followed-up at 40 months of age (n=49), investigators found that parents reported fewer attention problems and social withdrawal problems in the choline supplemented group compared to controls⁷⁷.

In contrast, another RCT which randomized women to consume supplemental choline (750 mg/d) or placebo from 18 weeks gestation through 90 days postpartum (n = 99) directly assessed a variety of cognitive domains in offspring including general cognitive function, language development, and episodic and

visuospatial memory (the study's primary focus)¹⁰. No benefit of maternal choline supplementation on infant cognitive functioning was detected¹⁰. However, this study may not have provided a strong test of the putative benefits of maternal choline supplementation on offspring cognition for several reasons. First, the visuospatial memory task (i.e., their task most consistent with hypotheses generated from rodent research) may not have provided a sensitive index of the infants' spatial memory ability. This concern arises due to reported low infant compliance and the consequent collapsing of infant performance data across all task conditions, precluding the opportunity to test for choline mediated improvements during the most difficult conditions of the task. Often, the effects of increased maternal choline intake in rodents are only observed under demanding conditions²². It is also possible that infants with the greatest spatial memory ability (putatively, infants exposed to prenatal choline supplementation) attempted a larger number of the difficult trials, potentially reducing their overall proportion correct relative to infants who had greater compliance on easy trials.

The final published RCT on the effect of maternal choline supplementation is unique in that the maternal diet and choline supplementation regimen were both highly controlled throughout the study⁷⁸. In this controlled feeding study, women in their 3rd trimester of pregnancy (n=24) were randomized to consume either 480 mg or 930 mg choline/d (approximately the AI and double the AI, respectively) for 12 weeks, holding all other aspects of the diet constant¹⁵. Because women consumed their choline supplement and at least one meal each weekday under study personnel supervision, high compliance was ensured¹⁵. Across the

first year of life (measurements at 4, 7, 10 and 13 months), infants whose mothers were randomized to the 930 mg/d group exhibited faster saccade reaction time during a test of visual attention compared to infants whose mothers were randomized to the 480 mg/d group, indicative of faster information processing speed⁷⁸.

1.5 Gaps in Knowledge

Strong evidence from rodents and inconsistent, but promising, findings from the human literature indicate that early-life exposure to choline, either prenatally or postnatally, influences offspring neurodevelopment and cognition. As noted earlier, choline plays an important role in early developmental processes as a precursor for the neurotransmitter acetylcholine, phospholipids such as phosphatidylcholine and sphingomyelin, and the methyl donor betaine. Consistent with these roles, decades of rodent research indicate that maternal choline intake has life-long effects on offspring cognitive functioning^{21,22}. Furthermore, research in humans suggest that the AI for choline, which few women currently consume, does not meet the demands of pregnancy⁷⁹. Despite this compelling data, remarkably few observational studies or RCTs in humans have evaluated whether maternal choline intake during pregnancy or lactation influences offspring cognition^{9–11,68,69,74,76–78}. Due in part to the important limitations of these studies outlined previously, the results of this research have so far been inconclusive.

The limitations of the existing research in humans reveal several important gaps in knowledge that will be addressed by this research. Notably, relatively few

studies have administered tests that directly assess child attention or memory, the two cognitive domains shown most consistently to be affected by maternal choline intake^{21,22}. Instead, many studies only administered tests of child intelligence or development which may not be sensitive to differences in maternal choline intake^{11,68,69}. Additionally, in two studies which reported positive results for electrophysiological measures related to attention and memory, no direct, behavioral measures of these domains were included^{74,76}. More research is needed to characterize the specific, behavioral effects of maternal choline supplementation on child cognitive functioning.

Most research on the relationship between maternal choline intake and child cognition has been conducted during infancy and early childhood, highlighting another important gap in knowledge. Specifically, the potential long-term effects of maternal choline supplementation on child cognition have yet to be thoroughly investigated. Only two observational studies^{9,68} and no RCTs have assessed child cognition during school-age. Importantly, as children age, measures of cognitive performance are more predictive of adult functioning. Assessments of child cognition extending later into childhood are needed to characterize the functional impact of maternal choline supplementation in the long-term.

A final gap in knowledge identified by surveying the available literature is the lack of research on the importance of maternal choline intake during lactation for child cognitive functioning. No RCTs have assessed whether maternal choline during lactation has beneficial effects on cognition in breastfed children. Only one observational study has been conducted in this area, assessing the association

between breastmilk free choline content and an electrophysiological measure of infant recognition memory⁷⁴. Importantly, although breastmilk free choline content was measured, maternal diet was not assessed; therefore, it is not known whether variation in maternal choline intake during lactation was an important predictor of infant cognitive function in this study. Research which characterizes the effects of maternal choline supplementation during breastfeeding on child cognitive functioning in the short- and long-term are needed.

1.6 Assessing the Effect of Prenatal or Postnatal Maternal Choline Intake on Child Cognition at Age Seven Years

The following dissertation papers describe the results of two, ancillary cognitive-behavioral follow-up studies of children born to women who participated in a randomized, controlled choline feeding study during either pregnancy or lactation^{15,80}. The first follow-up study assessed the effect of 3rd trimester choline supplementation on child cognition at 7 years of age (see **Figure 1.1**), and was motivated in-part by the beneficial effects of higher 3rd trimester choline intake on information processing speed detected in these same children during infancy⁷⁸. Briefly, 3rd trimester pregnant women were randomized to consume either 480 mg or 930 mg choline/d (approximately the AI and double the AI, respectively) beginning at gestational week 27 and ending at birth (approximately 12 weeks). The remainder of the women's nutrient intake was held constant at nutritionally adequate levels. At 7 years of age, children were invited to participate in a follow-up study to assess the effect of prenatal choline intake on child cognition.

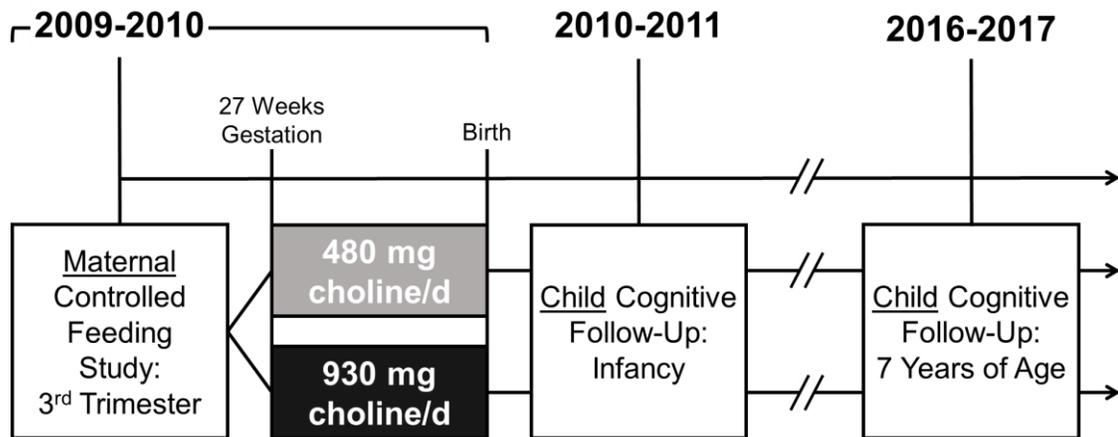


Figure 1.1 Study design and timeline of an ancillary follow-up to assess the effect of 3rd trimester choline supplementation on child cognitive functioning at age 7 years

The second follow-up study assessed the effect of maternal choline supplementation during lactation on child cognition at 7 years of age (see **Figure 1.2**). Briefly, postpartum women intending to exclusively breastfeed were randomized to consume either 480 mg or 930 mg choline/d (approximately the pregnancy AI and double the pregnancy AI, respectively) for 10 weeks beginning at 5 weeks postpartum. The remainder of the women’s nutrient intake was held constant at nutritionally adequate levels. At 7 years of age, children were invited to participate in a follow-up study to assess the effect of maternal choline intake during lactation on child cognition.

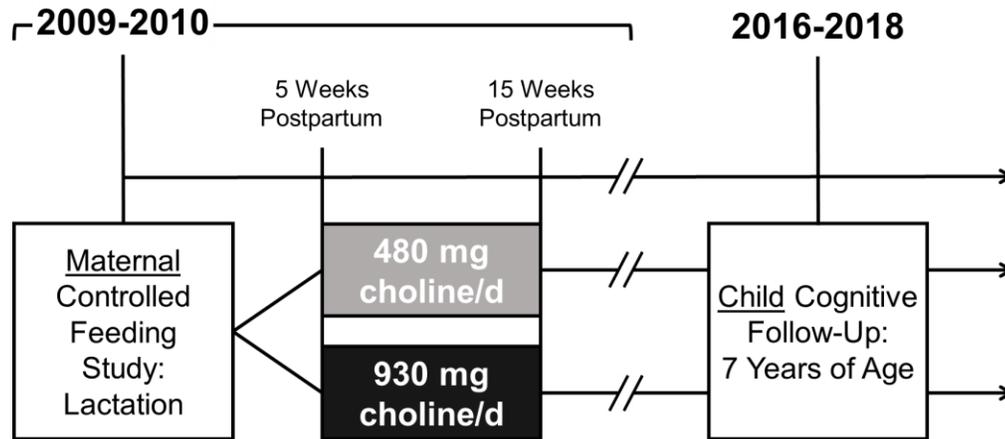


Figure 1.2 Study design and timeline of an ancillary follow-up to assess the effect of postnatal maternal choline supplementation on child cognitive functioning at age 7 years

For both of these follow-up studies, a battery of tasks was developed to evaluate child cognitive-behavioral functioning at 7 years of age, focusing specifically on tasks that assess the cognitive functions shown to be improved by maternal choline supplementation in rodents; namely, attention and spatial cognition^{21,22}. In addition, tests of cognitive functions that could plausibly be affected by maternal choline supplementation, such as executive functioning, and broader assessments of intelligence were administered. A description of the full testing protocol is presented in **Appendix A**.

This dissertation presents the results of three of the administered tasks for each study: 1) the sustained attention task (SAT)⁸¹, a signal detection task previously shown to be sensitive to manipulations in maternal choline intake in mice²⁷, 2) Mr. Peanut^{82,83}, a child-friendly assessment of hippocampal dependent memory, an aspect of cognition shown to be sensitive to maternal choline supplementation in rodents²¹, and 3) the Wechsler Preschool and Primary Scales of Intelligence, Fourth Edition (WPPSI-IV), a series of tasks which are used to

assess intelligence quotient (IQ) and other, more specific domains of cognitive functioning in children aged 2.5 years through 7.75 years of age⁸⁴. Findings from the study assessing the effect of prenatal choline supplementation are reported in **Chapter 2** (SAT) and **Chapter 3** (Mr. Peanut and WPPSI-IV). Results from the study assessing the effect of maternal choline supplementation during lactation are reported in **Chapter 4** (SAT, Mr. Peanut, and WPPSI-IV).

These two ancillary follow-up studies provide a unique opportunity to assess the long-term effects of maternal choline intake on child cognitive outcomes for several reasons. First, maternal choline intake during the intervention period was highly controlled. All food and supplements were provided by the study during the intervention period and high compliance was maintained by having women consume a meal and their choline supplement under study personnel supervision on weekdays. This ensured that meaningful differences between treatment groups in maternal choline intake during the intervention period were maintained. Second, the follow-up assessed specific aspects of memory and attention shown to be affected by differential maternal choline intake in rodent models. This provided a rigorous test of the hypothesized benefits of increased maternal choline intake in human^{21,22}. Finally, the ancillary follow-up studies were conducted when the children were 7 years of age, an age when more complex aspects of cognitive functioning can be measured compared to assessments during infancy or early childhood. Furthermore, performance on cognitive tests during the school-age years can predict later academic outcomes, providing potentially important insights into later functioning^{85,86}.

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CHAPTER 2: PRENATAL CHOLINE SUPPLEMENTATION IMPROVES CHILD ATTENTIONAL CONTROL: AN ANCILLARY 7-YEAR FOLLOW-UP OF A RANDOMIZED CONTROLLED FEEDING TRIAL

2.1 Abstract

Background: Numerous rodent studies have demonstrated developmental programming of offspring cognition by prenatal choline intake, with deprivation causing lasting adverse effects and supplementation producing lasting benefits. However, maternal choline intake levels needed to support optimal fetal neurodevelopment in humans is unknown and few studies have evaluated the effect of maternal choline supplementation on offspring cognition — none during the school-age years. **Objective:** To assess the effect of 3rd trimester maternal choline supplementation on child attention at age 7 y. **Design:** Third trimester pregnant women were randomized to consume either 480 mg choline/d (approximately the Adequate Intake [AI]) or 930 mg choline/d, from gestation wk 27 until delivery, as part of a controlled-feeding study. The present report represents a component of an ancillary study conducted to assess child cognition at age 7 y (n=20); here we report results from a signal detection task in which children indicated the presence or absence of a brief visual signal of variable duration on each of 216 trials. **Results:** For the primary outcome (SAT score), which combined performance on signal and non-signal trials, children whose mothers consumed 930 mg choline/d performed better than children in the 480 mg/d group (p=0.02)—an effect that didn't vary by signal duration. For correct signal detections (percent hits), the 930

mg/d group maintained performance across the session better than the 480 mg/d group ($p=0.02$), indicating superior sustained attention. Together, these findings indicate enhanced attentional control for the 930 mg/d group. **Conclusions:** Third trimester choline intake of 930 mg/d produced better child attentional control at age 7 y, relative to intake of 480 mg/d. These findings suggest that the choline AI for pregnancy is insufficient for optimal offspring cognitive functioning and raise concerns about choline intake during pregnancy in North America which, on average, is substantially less than the AI.

2.2 Introduction

Physiological demands for choline increase markedly during pregnancy^{1,2}, due to choline's numerous roles in fetal development. Specifically, choline is a precursor for: 1) phosphatidylcholine, a major component of biological membranes; 2) sphingomyelin, a primary constituent of myelin; 3) acetylcholine, a neurotransmitter and ontogenetic signal for regulating neurogenesis and synaptic plasticity^{3,4}; and 4) betaine, a methyl donor that (through DNA methylation) can exert life-long effects on gene expression⁵⁻⁷. Consistent with these roles, over 25 years of research in rodents has demonstrated the importance of maternal choline intake for the developmental programming of offspring cognition. Specifically, maternal choline deprivation produces lasting offspring cognitive impairment^{8,9}, whereas prenatal choline supplementation improves offspring spatial memory and attention and protects against aging-related cognitive decline⁸⁻¹⁵.

Despite a large body of rodent research, little is known about the functional

effects of maternal choline intake on offspring cognition in humans and the maternal intake level needed to fully support fetal neurodevelopment is poorly defined. An Adequate Intake (AI) for pregnant women (450 mg/d) was established in 1998; however, this value was extrapolated from evidence pertaining to the amount of choline needed to prevent liver dysfunction in men and not on measures of offspring functioning¹⁶. The few studies that have evaluated the effects of maternal choline intake on offspring cognition in humans are inconclusive^{17–23}. Two observational studies found that greater concentrations of choline metabolites in maternal plasma¹⁷ or greater estimated prenatal dietary choline intake¹⁸ was positively associated with child performance on cognitive tests; but two other observational studies found no association^{19,20}. Only three randomized controlled trials (RCTs) have explored this topic. One trial focused on infant memory and did not detect benefits²¹. The two other trials reported beneficial effects of maternal choline supplementation, one on infant information processing speed²² and the other on an electrophysiological index of cerebral inhibition²³; both results may reflect improved attention. Notably, no RCTs of maternal choline supplementation have followed children into school age, a time when complex cognitive functioning can be measured.

To address this need, the present study leveraged a controlled choline feeding trial in which pregnant women had been randomized to one of two levels of choline intake during the third trimester. Offspring information processing speed was assessed during infancy²², as noted above. We followed-up these children at age 7 y to assess cognitive performance; the present report describes

the results of a signal detection task assessing attentional function.

2.3 Subjects and Methods

Ethical Approval

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 and child assent was obtained from all study participants.

Study Design & Participants

The present study is a 7-y follow-up of the children born to women who participated in a randomized, double-blind, parallel-group controlled choline feeding study during their third trimester of pregnancy (NCT01127022). The feeding study was powered to assess the primary outcomes of maternal/fetal biomarkers of choline metabolism^{1,24}. Secondary outcomes included genomic expression, metabolomic profiling of plasma and placental tissue^{5,25}, and offspring cognition during infancy²². The present study is an ancillary follow-up of the children at age 7 y to test for effects on child cognition, using prespecified primary and secondary outcomes.

Details of the controlled feeding study, including the study diet, have been published elsewhere¹. Briefly, 3rd trimester pregnant women (27 wk gestation) aged ≥ 21 y were recruited from the Ithaca, NY region in 2008-2009. Eligibility for the study was contingent on a variety of factors including general good health and willingness to comply with the study protocol. Exclusion criteria included: 1)

use of alcohol or tobacco products during pregnancy, 2) non-singleton pregnancy, and 3) pregnancy-related complications such as preeclampsia, gestational diabetes, or intrauterine growth restriction.

Women were randomized to consume either 480 mg choline/d (approx. the AI) or 930 mg choline/d from enrollment until delivery (approx. 12 wk). To achieve these total choline intake levels, all women consumed the study diet providing 380 mg choline/d and an additional choline supplement of either 100 or 550 mg choline/d. The choline supplement (choline chloride, Balchem Corp.) was mixed with cran-grape juice and served in color-coded tubes such that participants and investigators were blinded to dose. No adverse effects of either choline dose were reported for the controlled feeding study. On weekdays, women consumed one meal/d and the choline supplement under supervision of study personnel in the Human Metabolic Research Unit at Cornell University. All other meals were provided as take-aways and consumed off site; participants were instructed to consume the choline supplement with a meal of their choice on weekend days. Compliance to the study diet and choline supplement was high based on in-lab monitoring of supplement and food consumption, return of supplement and food containers for weekend days, and greater fasting plasma concentrations of choline and its metabolites in the 930 mg/d (v. 480 mg/d) group¹. In addition to the study diet and choline supplement, all women consumed a daily prenatal multivitamin (Pregnancy Plus, Fairhaven Health LLC), a daily 200 mg docosahexaenoic acid (DHA; Neuromins, Nature's Way Products), and a thrice weekly 250 mg potassium and 250 mg magnesium supplement (General

Nutrition Corp.).

Between August 2016 and March 2017, children born to the women enrolled in the controlled choline feeding study were invited to participate in a 7-year follow-up to investigate the effect of 3rd trimester choline intake on child cognitive functioning. Children were tested between 7.0 and 7.7 y of age at Cornell University (n=16), or at an alternate location if travel to Ithaca was not possible (n=4). Characteristics of the participants and their mothers were obtained via parent report at the time of follow-up and included child age, sex, visual acuity, grade in school, race, ethnicity, and maternal age at child conception and current educational attainment. Maternal characteristics at the time of the feeding study, including race, ethnicity, education, and age, were evaluated to assess bias arising from loss to follow-up.

Assessment of Attention

Children were administered a challenging signal detection task (referred to as the “Sustained Attention Task” [SAT])²⁶ by one of two trained study personnel who were blinded to group assignment, as part of a two-day cognitive testing protocol. The SAT was selected because maternal choline supplementation in mice was found to improve offspring attentional performance on a rodent analog of this task²⁷. A detailed description of the SAT is presented in **Figure 2.1**. Briefly, for each trial of the SAT, the child was required to indicate whether s/he saw or did not see a signal (a small, low-contrast gray square) presented for a variable duration (17, 29, or 50 ms) on a light gray background. A signal was presented randomly on 50% of the 216 total trials, with an equal number of signal and non-

signal trials occurring every 18 trials. An auditory cue, presented 100 ms after the signal or non-signal event, prompted the child to indicate whether s/he saw or did not see a signal by pressing the appropriate, pre-specified key on the testing laptop. The child was allowed 1500 ms to make a response. If the child made a correct response, a 500 ms tone was presented. If the child made an incorrect response or made no response, no tone was presented.

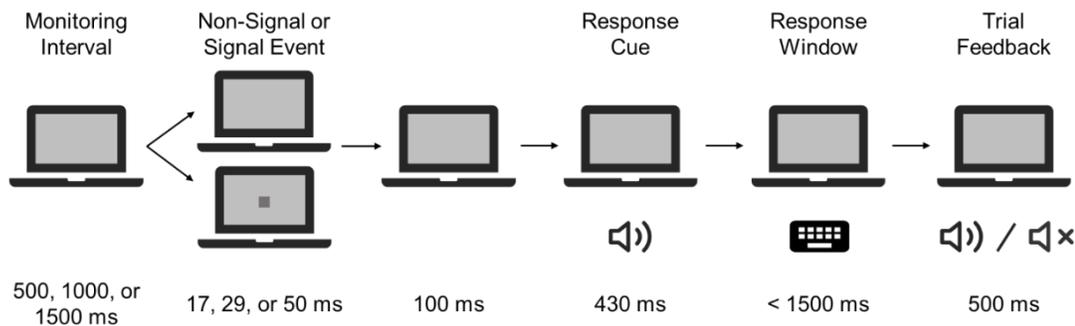


Figure 2.1. Sustained Attention Task (SAT). The SAT consisted of 216 trials in which the child was instructed to indicate whether they saw or did not see a brief, low-contrast signal (a 5 mm x 5 mm gray square) of variable duration (17 ms, 29 ms, or 50 ms) on a light gray screen. Each trial began with a variable monitoring interval (500 ms, 1000 ms, or 1500 ms) to prevent anticipatory responding. Following the monitoring interval, a non-signal or signal event occurred. A signal was presented randomly on 50% of the trials, with an equal number of signal and non-signal trials occurring every 18 trials. One hundred ms after the signal or non-signal event occurred, a 430 ms auditory response cue (“Go”) indicated the opening of the response window; i.e., that it was time to respond. Children had 1500 ms to indicate whether they saw or did not see a signal by pressing the appropriate, pre-specified key on the laptop keyboard (key assignments were determined by child handedness). Correct responses were followed by a 500 ms positively-valenced tone. No feedback was given after incorrect responses or omissions.

Each child was administered a minimum of 12 practice trials to demonstrate understanding of task rules and procedures. After a child displayed satisfactory understanding, the full SAT was administered (approx. 11.5 min in

duration). The SAT was programmed in OpenSesame Version 3.0.4 and administered on a 30.48 cm x 19.05 cm Dell laptop²⁸. Responses were categorized as hits, misses, correct rejections, false alarms, and omissions, as defined in **Table 2.1**.

Table 2.1 SAT trial outcomes defined by trial type and child response

Trial Type	Child Response		
	<i>Saw Signal</i>	<i>Did Not See Signal</i>	<i>No Response</i>
<i>Signal Trial</i>	Hit	Miss	Omission
<i>Non-Signal Trial</i>	False Alarm	Correct Rejection	

Performance on the SAT depends on both sensory detection ability (e.g., visual acuity) and attentional control processes^{29,30}. Attentional control refers to cognitive functions which allow an individual to achieve and maintain a high level of task performance, such as preventing lapses in attention, sustaining attentional focus, and suppressing behaviors incompatible with accurate performance³¹. The primary outcome, SAT score, measures the ability to accurately identify the presence and absence of the signals. SAT score is a composite score that integrates performance across signal and non-signal trials; it is calculated using the following formula:

$$\frac{\text{Percent Hits} - \text{Percent False Alarms}}{2(\text{Percent Hits} + \text{Percent False Alarms}) - (\text{Percent Hits} + \text{Percent False Alarms})^2}$$

, where *Percent Hits* = $\text{Hits} \div (\text{Hits} + \text{Misses})$ and *Percent False Alarms* = False

Alarms \div (*False Alarms* + *Correct Rejections*). SAT score ranges from -1 to +1. Scores ≤ 0 represent an inability to discriminate signal from non-signal trials; a score of +1 indicates perfect responding²⁶. We also analyzed percent hits and percent false alarms, the primary components of SAT score, to provide insight into group differences not discernible from this composite score. In addition, omission errors were analyzed to assess task engagement.

Video Coding

Video recordings of children's behaviors during testing were coded to identify instances of off-screen looking. Each video was independently coded by two trained study personnel blinded to group assignment using Behavioral Observation Research Interactive Software Version 4.1.4³². Trials missed due to off-screen looking were identified and total duration of off-screen looking was calculated for each participant.

Statistical Analysis

Maternal and child characteristics for the two treatment groups in the final analytic sample were compared using Student's *t* tests for continuous variables and chi-square tests for categorical variables, as were potential differences between the final analytic sample and participants lost to follow-up or excluded from analysis.

Performance on the SAT was modeled as a function of task block (3 blocks of 72 trials) and signal duration (as applicable). Linear mixed models were used to assess the effect of 3rd trimester choline intake on SAT score, percent hits, and percent false alarms, and a generalized linear mixed model with a binomial

distribution was used to assess omissions. All mixed models included random effects of child and task block within child. Child sex was included as a fixed effect in all mixed models; due to research which suggests that child performance on several tests of attention varies by sex³³⁻³⁷ and limited power to adjust for other covariates, child sex was specified *a priori* as the sole covariate^{38,39}. Analyses for SAT score, percent hits, and omissions included the three-way interaction between treatment group, task block, and signal duration and corresponding two-way interactions; analyses for percent false alarms included the two-way interaction between treatment group and task block. Treatment group differences in performance across task blocks were evaluated using planned, single degree of freedom linear and quadratic interaction contrasts⁴⁰. Additionally, group differences in time spent looking off-screen were assessed using a Wilcoxon rank sum test. Finally, sensitivity analyses were conducted to evaluate the possible influence of trials missed due to off-screen looking.

Statistical tests were two-sided with $p < 0.05$ indicating statistical significance for main effects, interactions, and planned contrasts. Pairwise comparisons were adjusted for multiplicity using a Bonferroni correction and considered statistically significant at $p < 0.05$. All data were analyzed using SAS version 9.4 (SAS Institute Inc., Cary, NC).

2.4 Results

Subject Characteristics

Of the 26 children whose mothers completed the randomized choline

feeding trial, 21 were re-recruited and 20 were successfully tested for the 7-year cognitive follow-up (77% retention; see **Figure 2.2**). One child did not comply with the cognitive testing protocol and did not contribute data to analyses; this determination was made prior to unblinding the data analyst to group assignment. Children included in the final analytic sample did not differ from children lost to follow-up or excluded from analyses on a variety of measures including child sex, maternal race/ethnicity, and maternal age and education level at conception (all $p \geq 0.27$; data not shown).

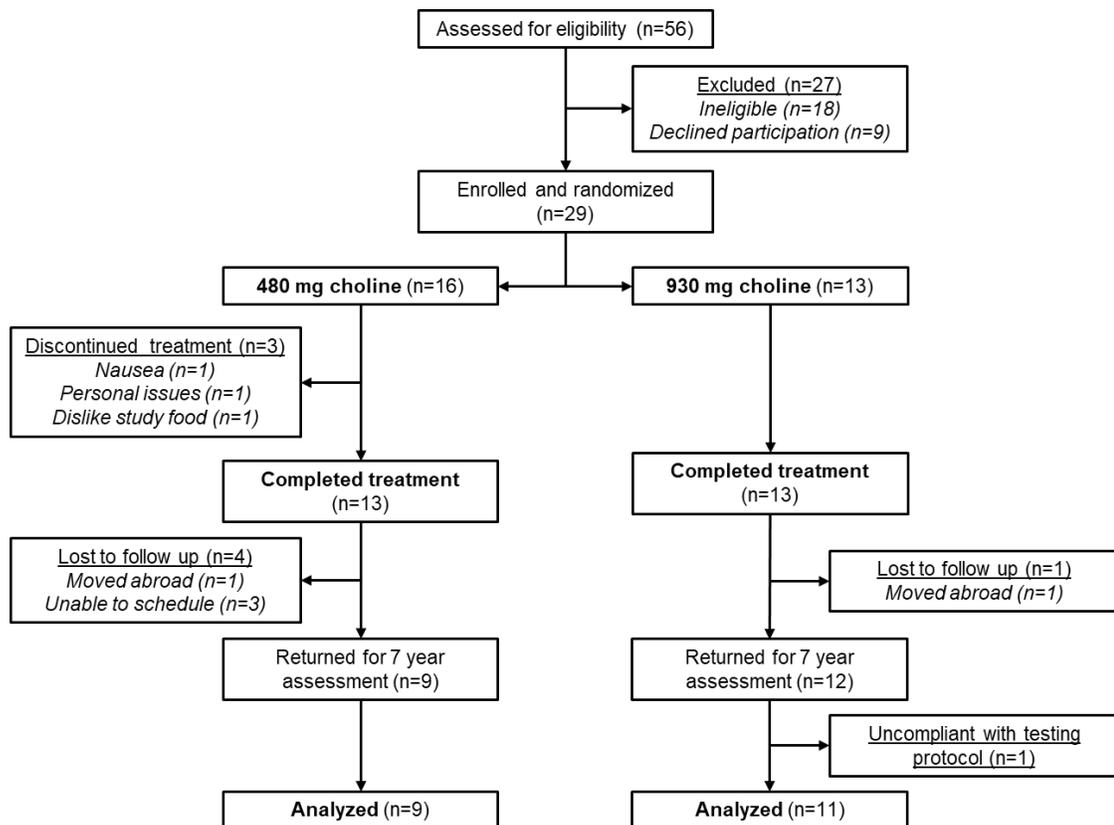


Figure 2.2 Participant flowchart for the assessment of the effect of 3rd trimester choline supplementation on child sustained attention task (SAT) performance at age 7 y

Demographic characteristics of the participants and their mothers are presented in **Table 2.2**. The child participants were predominantly non-Hispanic white and male, and most had completed 1st grade. Mothers of the children were mostly highly educated — the majority having earned a bachelor's degree or higher. Although the treatment groups did not differ significantly on any background variable, mothers of children in the 480 mg choline/d group tended to have a higher level of educational attainment than mothers of children in the 930 mg choline/d group.

Table 2.2 Select characteristics of children included in final analytic sample¹

	Third trimester maternal choline intake		p ²
	480 mg/d (n=9)	930 mg/d (n=11)	
Child characteristics			
Sex (male)	6 (67)	8 (73)	0.77
Mean age in y (SEM)	7.2 (0.2)	7.3 (0.2)	0.68
Highest grade completed			0.44
<i>Kindergarten</i>	3 (33)	2 (18)	
<i>First Grade</i>	6 (67)	9 (82)	
Normal or corrected to normal vision	9 (100)	11 (100)	NA
Race			0.53
<i>Asian</i>	1 (11)	2 (18)	
<i>Black</i>	0 (0)	1 (9)	
<i>Native American</i>	0 (0)	1 (9)	
<i>White</i>	8 (89)	7 (64)	
Hispanic	2 (22)	2 (18)	0.82
Maternal characteristics			
Education			0.08
<i>High School / Associates degree</i>	0 (0)	4 (36)	
<i>Bachelors degree</i>	3 (33)	4 (36)	
<i>Masters / Doctoral degree</i>	6 (67)	3 (28)	
Mean age at conception in y (SEM)	28.4 (3.0)	27.6 (3.7)	0.61

¹All values n (%) unless otherwise noted. ²Reported p-values from Student's t test (continuous variables) and chi-square tests (categorical variables)

SAT Score

Task design effects: The main effect of signal duration was statistically significant ($F_{(2,108)} = 17.17$, $p < 0.0001$), such that SAT score was lower for the briefest cues. Specifically, performance on 17 ms trials was lower than on 29 ms ($t_{(108)} = 5.18$, 95% CI [-0.19, -0.09], adjusted $p < 0.0001$) and on 50 ms trials ($t_{(108)} = 4.96$, 95% CI [-0.19, -0.8], adjusted $p < 0.0001$). SAT score did not differ

between 29 ms and 50 ms trials ($t_{(108)} = 0.22$, 95% CI [-0.05, 0.06], adjusted $p > 0.99$). A main effect of task block was not detected ($F_{(2,36)} = 1.52$, $p = 0.23$).

Maternal choline effects: The main effect of treatment group on SAT score was significant; children whose mothers consumed 930 mg choline/d during the 3rd trimester of pregnancy obtained higher SAT scores than children whose mothers consumed 480 mg choline/d ($F_{(1,17)} = 7.19$, 95% CI [0.03, 0.27], $p = 0.02$; see **Figure 2.3**). The effect of treatment group did not vary by signal duration ($F_{(2,108)} = 0.30$, $p = 0.74$). Furthermore, linear change in performance across the testing session did not vary by treatment group ($t_{(36)} = 0.44$, $p = 0.66$). No three-way interaction was detected for SAT score or for any other outcome (all $p \geq 0.43$; not shown).

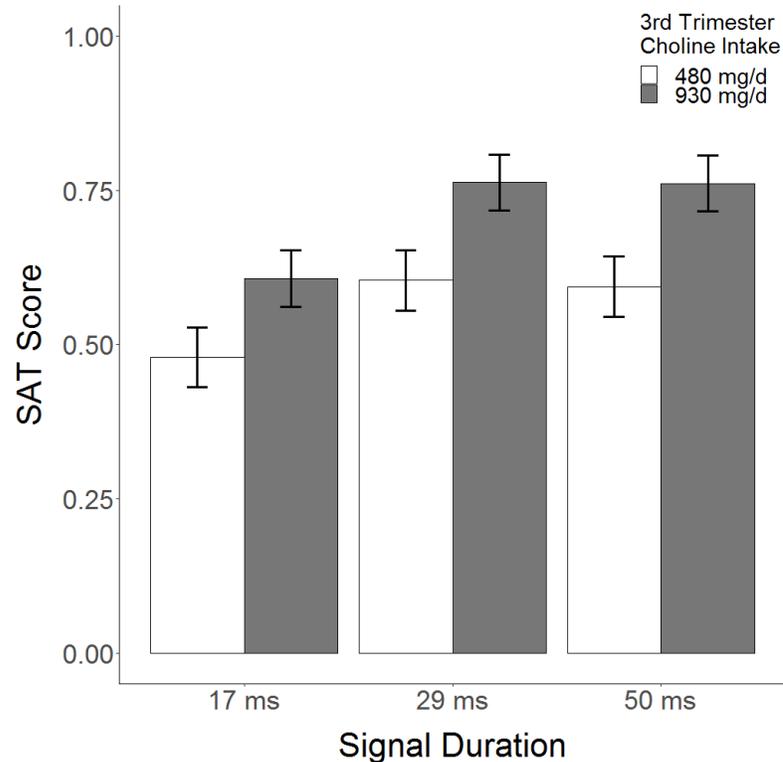


Figure 2.3 Effect of 3rd trimester choline intake and signal duration on SAT score. Children in the 930 mg/d group had a higher average SAT score than children in the 480 mg/d group (main effect of treatment group: $p = 0.02$). SAT score differed significantly by signal duration ($p < 0.0001$), but the 3rd trimester choline intake by signal duration interaction was non-significant ($p = 0.74$). Values represent least square means \pm SEM. 480 mg/d: $n = 9$; 930 mg/d: $n = 11$.

Percent Hits

Task design effects: A main effect of signal duration ($F_{(2,108)} = 29.63$, $p < 0.0001$) revealed that percent hits was lowest for the briefest cue: percent hits for 17 ms trials was significantly lower than for 29 ms trials ($t_{(108)} = 6.93$, 95% CI [-21%, -12%], adjusted $p < 0.0001$) and for 50 ms trials ($t_{(108)} = 6.36$, 95% CI [-20%, -11%], adjusted $p < 0.0001$). Percent hits did not differ between 29 ms and 50 ms trials ($t_{(108)} = 0.57$, 95% CI [-34%, 6%], adjusted $p > 0.99$).

Maternal choline effects: The main effect of treatment group on percent

hits was not significant ($F_{(1,17)} = 2.62$, 95% CI [-2%, 17%], $p = 0.12$), nor was the interaction between treatment group and signal duration ($F_{(2,108)} = 0.49$, $p = 0.61$). However, the linear change in performance across the testing session differed significantly between the two treatment groups ($t_{(36)} = 2.37$, 95% CI [2%, 27%], $p = 0.02$) and no quadratic trend was indicated ($t_{(36)} = 0.85$, $p = 0.40$). As shown in **Figure 2.4**, children whose mothers consumed 480 mg choline/d during the 3rd trimester exhibited a significant decline in percent hits across the session ($t_{(36)} = 3.54$, 95% CI [-25%, -7%], adjusted $p = 0.002$), whereas children whose mothers consumed 930 mg choline/d exhibited no change in percent hits across the session ($t_{(36)} = 0.38$, 95% CI [-10%, 7%], adjusted $p > 0.99$).

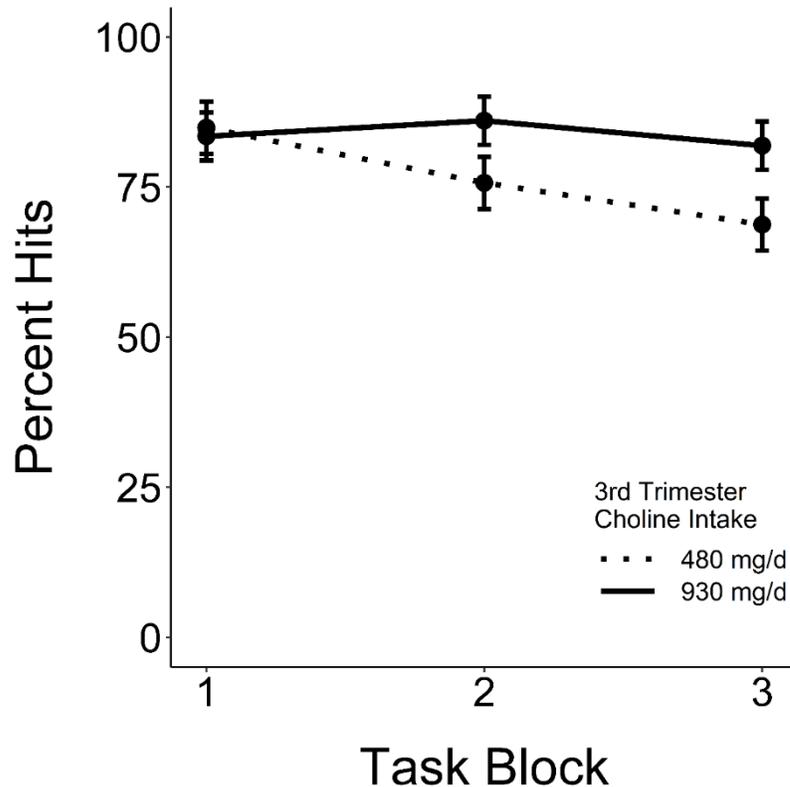


Figure 2.4 Effect of 3rd trimester choline intake and task block on percent hits. Linear change in percent hits (correct signal detections) across the task blocks varied by 3rd trimester choline intake ($p = 0.02$): the 480 mg/d group exhibited significant decline across blocks ($p = 0.002$) whereas the 930 mg/d group exhibited no change in performance across blocks ($p > 0.99$). Values represent least square means \pm SEM. 480 mg/d: $n = 9$; 930 mg/d: $n = 11$.

Percent False Alarms

Task design effects: Percent false alarms did not vary by task block ($F_{(2,36)} = 0.40$, $p = 0.67$).

Maternal choline effects: Percent false alarms did not vary by maternal choline intake ($F_{(1,17)} = 1.21$, 95% CI [-22%, 7%], $p = 0.29$). The linear change in false alarm rate across the session did differ by treatment group ($t_{(36)} = 2.07$, 95% CI [0.3%, 25%], $p = 0.045$), but neither group showed significant change across blocks and the groups did not differ at any individual test block (all adjusted $p \geq$

0.43; data not shown).

Omissions

Task design effects: The overall omission rate was low (Median: 3.7%, IQR: 2.1% – 13.4%), indicating that children were engaged with the task. Whereas no effect of task block was detected ($F_{(2,92.13)} = 0.25$, $p = 0.78$), the omission rate did vary by signal duration ($F_{(3,215)} = 53.01$, $p < 0.0001$; LS Mean [SEM]: non-signal, 6.2% [2.6]; 17 ms, 2.0% [0.9]; 29 ms, 0.6% [0.3]; 50 ms, 0.6% [0.3]). Specifically, omissions were more likely during non-signal trials than for signal trials of any duration (17 ms: $t_{(215)} = 6.57$, 29 ms: $t_{(215)} = 8.42$, 50 ms: $t_{(215)} = 8.42$; all adjusted $p < 0.0001$). For signal trials, omissions occurred more frequently during trials with the briefest cues. Specifically, the incidence of omission errors was greater for trials with a 17 ms cue than for those with a 29 or 50 ms cue (29 ms: $t_{(215)} = 3.85$, 50 ms: $t_{(215)} = 4.01$; both adjusted $p < 0.001$); omissions on 29 ms and 50 ms trials did not differ ($t_{(215)} = 0.22$, adjusted $p > 0.99$).

Maternal choline effects: The omission rate did not differ between choline groups ($F_{(1,17.06)} = 0.54$, $p = 0.47$), and there were no statistically significant interactions between any design variables for this outcome (all $p \geq 0.26$; data not shown).

Off-Screen Looking

Of the 20 participants, 19 had video recordings of the SAT available for behavioral coding (930 mg/d: $n = 10$, 480 mg/d: $n = 9$). Review of the videos revealed that children were consistently engaged with the task, with only 0.4% of trials (17 out of 4099) missed due to off-screen looking. Only nine of the 19

participants with available data looked off screen at least once during the task; this proportion did not differ between treatment groups (Fisher's exact test: $p = 0.66$). The median amount of time spent looking off-screen was 0 s (IQR: 0 s – 4.7 s) and did not differ significantly between choline groups ($Z = 0.66$, $p = 0.51$). Sensitivity analyses which excluded trials missed due to off-screen looking in no case altered conclusions (see **Appendix B**).

2.5 Discussion

In this follow-up of a randomized controlled choline feeding study, 7 y old children whose mothers consumed 930 mg choline/d during their 3rd trimester performed significantly better on a challenging signal detection task than children whose mothers consumed 480 mg choline/d. Children in the 930 mg/d group achieved higher SAT scores than those in the 480 mg/d group, an effect that did not interact with signal duration. The pattern of results is most consistent with the inference that groups differed in attentional control, based on several lines of reasoning. First, it is unlikely that higher prenatal choline intake enhanced sensory processing of the brief visual signals; such an effect would have likely produced an interaction between choline group and signal duration, with the greatest benefit seen at the briefest signal. Second, we found no evidence for group differences in motivation, which theoretically could have produced the observed pattern of results. Specifically, groups did not differ in either omissions or amount of off-screen looking. Instead, the observed pattern of results is consistent with a group difference in the incidence of brief lapses in attention, a core facet of

attentional control⁴¹. Because attentional lapses generally last much longer than 50 ms⁴² (the longest cue duration studied in the present task), their effect on SAT performance would be expected to similarly affect all signal trials, regardless of duration, consistent with observed results.

This inference of superior attentional control in the 930 mg/d group is also consistent with the observed pattern of results for signal trials as well as the absence of treatment effects for non-signal trials. Change in percent hits across the testing session varied by treatment group: The 480 mg/d group exhibited a significant decline across the session, whereas the 930 mg/d group maintained stable performance. This pattern of results suggests that the ability to sustain attention, another core facet of attentional control⁴³, was superior for children in the 930 mg/d group. The absence of group differences for non-signal trials is also consistent with the conclusion that maternal choline intake affected child attentional control because performance on these trials is unlikely to be influenced by attentional lapses. Specifically, if an attentional lapse occurred during a non-signal trial, the child would still likely respond that a signal had not appeared, and the response would be recorded as correct.

The finding of increased attentional control in children born to women in the higher choline group is consistent with prior rodent findings. One prior study reported that maternal choline supplementation during pregnancy improved offspring performance on a murine analog of the SAT²⁷. The pattern of effects in mice was similar to that seen in the present study: a benefit was observed for signal trials, but not for non-signal trials. Several other rodent studies have

reported that maternal choline supplementation during pregnancy improves offspring attention in other types of tasks^{12–14,44}.

Although few human studies have assessed the effect of maternal choline supplementation on child cognition, our findings of improved attentional control are consistent with two prior reports. First, they concur with the results of a visual reaction time test administered during infancy to the children in the present cohort. Specifically, infants born to women in the 930 mg/d group (v. 480 mg/d group) exhibited faster attentional orienting speed, a possible developmental precursor to improved attentional control^{22,45}. Second, in a study by Ross and colleagues²³, 5 wk old infants whose mothers were randomized to take 900 mg supplemental choline/d (v. placebo) during pregnancy displayed improved cerebral inhibition, an electrophysiological measure associated with reduced risk of attentional disorders later in life²³. No prior intervention studies have evaluated the effects of maternal choline supplementation on offspring cognition during school-age. However, two observational studies have evaluated links between maternal choline intake or plasma choline metabolite concentrations and cognition during the school-age years; one reported a positive association with memory performance at 7 y of age¹⁸, and one found no association with a global test of IQ at 5 y of age¹⁹.

Little is known about the brain changes that may mediate the observed improvements in attentional function in these studies. Rodent studies have observed changes to cholinergic neurons in the basal forebrain due to maternal choline supplementation^{46–48}; it is possible that our intervention altered basal

forebrain cholinergic neurons projecting to the prefrontal cortex, an area which modulates attentional control^{43,49,50}.

The present study has several strengths. First, the study design allows for strong causal inferences. All food and choline supplements were provided by the study and participants consumed more than 70% of the choline supplements under study personnel supervision, ensuring substantial group differences in choline intake. Second, video coding and analysis of omissions enabled us to rule out group differences in off-task behavior or motivation as the basis of treatment effects on performance. A final strength of the study was the use of a task previously used to assess the effects of maternal choline supplementation in rodents. The demonstration that maternal choline supplementation produces a similar pattern of effects in homologous tasks in rodents and humans suggests that the numerous offspring benefits observed in rodents may translate to humans. These benefits include a lessening of cognitive impairment in diverse conditions including aging¹¹, Down syndrome^{12,13,46,51}, autism⁵², and Alzheimer's disease⁵³.

However, this study also has limitations. First, the sample size was small, increasing the risk of chance findings⁵⁴. We emphasize, however, that the outcomes selected for this task and the statistical models used to assess them were specified *a priori*, thus reducing our exposure to Type 1 errors. Second, the relatively homogenous nature of the sample (e.g., predominantly white, male, high maternal education level) may limit the generalizability of our findings. Finally, when interpreting these findings one must note that choline intake levels for both groups in this study are greater than the average consumption of pregnant

women in North America (approx. 350 mg/d)^{18,55–58}; it would have been unethical to feed pregnant women a total dietary choline intake less than the AI. As a result, our data do not directly address the effect of increasing maternal choline intake from current average levels to either level administered in the study.

In conclusion, higher 3rd trimester choline intake resulted in superior offspring attentional control at age 7 y. Attentional control contributes to a variety of functions such as problem-solving and working memory, and is positively associated with school performance, supporting the real-world significance of these results^{59–63}. The findings from this cohort at age 7 y extend our results from infancy²² and provide new evidence that maternal choline supplementation during pregnancy has lasting benefits on offspring cognition. In fact, because consumption of 930 mg choline/d produced superior child cognition relative to consumption of approximately the AI, these findings suggest that the choline AI for pregnant women may not be sufficient for optimal child cognition--although replication in a larger clinical trial is needed. These findings also raise concerns about the evidence that approximately 90% of pregnant women in the US consume choline at levels below the AI and that most prenatal vitamins contain little or no choline⁶⁴.

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CHAPTER 3: EFFECT OF THIRD-TRIMESTER CHOLINE SUPPLEMENTATION ON CHILD MEMORY AND INTELLIGENCE: A 7-YEAR FOLLOW-UP OF A RANDOMIZED CONTROLLED FEEDING STUDY

3.1 Abstract

Background: Thirty years of rodent research have demonstrated that prenatal choline availability affects offspring cognitive functioning, most notably spatial cognition and memory. However, prenatal choline intake levels needed to support optimal fetal neurodevelopment in humans are unknown and few human studies have evaluated the effect of prenatal choline supplementation on child cognition.

Objective: To assess the effect of 3rd trimester choline supplementation on child memory and domains of intelligence at age 7 y. **Design:** Pregnant women (n=26) were randomized to consume either 480 mg choline/d (approx. the Adequate Intake [AI]) or 930 mg choline/d from gestational week 27 until delivery as part of a controlled-feeding study. An ancillary follow-up study assessed offspring cognition at age 7 y. The present report describes performance on a visuospatial short-term memory task, in which the locations of colored dots on a cartoon figure were recalled after a retention interval of 1 s or 8 s, and the Weschler Preschool and Primary Scales of Intelligence (WPPSI-IV). **Results:** The maximum number of dots recalled was greater for children in the 930 mg/d group than those in the 480 mg/d group ($p=0.048$), indicative of better visuospatial short-term memory capacity. This effect was primarily due to superior performance by the 930 mg/d group at the 1 s RI compared to the 480 mg/d group ($p=0.052$). WPPSI-IV performance did not vary significantly by treatment group for any outcome assessed, but the

pattern of mean differences was consistent with previous research documenting benefits of maternal choline supplementation on offspring memory and attention.

Conclusions: Third trimester choline intake of 930 mg/d produced better visuospatial short-term memory capacity in children compared to 480 mg/d (approx. the AI). These findings suggest that the choline AI for pregnant women may not be sufficient for optimal offspring memory.

3.2 Introduction

Three decades of rodent research has shown that prenatal choline availability influences fetal brain development and life-long neurobehavioral functioning of offspring^{1,2}. Specifically, maternal choline deficiency produces lasting offspring cognitive impairment^{1,3}, whereas maternal choline supplementation improves offspring attention and memory^{1,2}, and also reduces cognitive dysfunction in models of aging⁴, Down syndrome⁵⁻⁸, and Alzheimer's disease⁹. The most robust effects of maternal choline supplementation have been demonstrated using tests of spatial memory performance^{1,2}. Consistent with this, prenatal choline supplementation produces structural^{8,10}, electrophysiological¹¹⁻¹³, and neurochemical¹⁴ changes within the hippocampus of offspring that indicate improved functioning of this critically important memory structure.

The functional effects of maternal choline intake on child cognition are not well characterized in humans and it remains unknown whether current intake recommendations (the Adequate Intake [AI]) meet the demands of fetal neurodevelopment. The few studies that have been conducted in this area have so far

provided inconclusive results, consisting primarily of observational studies which assess broad aspects of infant and child cognition. One study found that 1st trimester maternal plasma free choline was associated with infant cognitive performance¹⁵, but three others found no association between measures of prenatal choline exposure and child intelligence test performance^{16–18}. However, it is likely that global tests of cognition are not as sensitive to variations in maternal choline intake as tests which measure the specific aspects of memory and attention shown to be affected by prenatal choline supplementation in rodents. Indeed, two randomized controlled trials (RCTs) detected beneficial effects of prenatal choline supplementation on child attention-related endpoints^{19,20}, and one observational study reported that 2nd trimester choline intake was positively associated with offspring visual memory at age 7 y¹⁸. However, no benefit of maternal choline supplementation was found in an RCT designed to assess infant visuospatial short-term memory and episodic memory²¹.

Notably, only two observational studies^{16,18}, and no RCTs, have assessed the relationship between prenatal choline exposure and child cognition into school age, a time when complex cognitive functions can be assessed. To address this knowledge gap and the general paucity of research in this area, we conducted a follow-up of a unique cohort of 7 y old children whose mothers participated in a randomized controlled choline feeding study during pregnancy²². The follow-up assessed a variety of cognitive and behavioral functions; the present report describes the results from tests of visuospatial short-term memory and intelligence.

3.3 Methods

Study Design & Participants

The present study is a 7-y follow-up of children born to women who participated in a randomized, double-blind, parallel-group controlled choline feeding study during the third trimester of pregnancy (NCT01127022), conducted at Cornell University (Ithaca, NY, USA). The primary outcome of the feeding study, for which the study was powered for, was maternal biomarkers of choline status^{22,23}. Secondary outcomes included genomic expression and metabolomic profiling of plasma and placental tissue^{24,25}, and offspring cognitive functioning during infancy¹⁹. The present study is an ancillary follow-up to assess the effect of prenatal choline intake on child cognitive outcomes at 7 y of age.

Details of the controlled choline feeding study, including study diet, have been published elsewhere²². Briefly, in 2008-2009, 3rd trimester pregnant women aged ≥ 21 years were recruited from Ithaca, NY and the surrounding areas. Study eligibility was contingent on a variety of factors including being in general good health and a willingness to comply with the study diet and protocol. Exclusion criteria included abnormal kidney or liver function, history of chronic disease, use of alcohol or tobacco products during pregnancy, non-singleton pregnancy, and pregnancy-related conditions such as preeclampsia, gestational diabetes, and intrauterine growth restriction.

Women enrolled in the study were randomized to consume either 480 or 930 mg choline/d from enrollment at 27 weeks gestation to delivery (approx. 12 weeks). All participants consumed the standard study diet providing, on average,

380 mg choline/d and a choline supplement providing 100 or 550 mg choline/d based on group assignment to achieve the target choline dose. The choline supplement (choline chloride, Balchem) was prepared by study personnel and served to participants mixed with cran-grape juice in color-coded tubes so that investigators and participants were blinded to dose. On weekdays, women consumed one meal/d at the Human Metabolic Research Unit (HMRU) at Cornell University and took their choline supplement on site while under the supervision of study personnel. Remaining weekday meals and all weekend meals were provided to participants and consumed off site. Participants were provided choline supplements for weekends and instructed to consume the choline supplement with a meal. Study compliance was high based on in-lab monitoring of food and supplement consumption and return of containers for meals and supplements consumed off-site. Additionally, women in the 930 (v. 480) mg/d group had significantly higher fasting levels of plasma choline and its metabolites²², consistent with reports of high compliance.

Participants also consumed a daily prenatal multivitamin (Pregnancy Plus, Fairhaven Health LLC) and 200 mg docosahexaenoic acid supplement (Neuromins, Nature's Way Products), and a thrice weekly potassium and magnesium supplement (250 mg/dose each; General Nutrition Corp.). Supplements were consumed under the supervision of study personnel at the HMRU on weekdays and with an off-site meal on weekends.

Children born to the women who participated in the controlled choline feeding study were invited to participate in an ancillary, 7-y follow-up to

investigate the effect of 3rd trimester choline on child cognitive and behavioral functioning. Child cognition was assessed over two, 90-min sessions by study personnel blinded to group assignment between August 2016 and March 2017 at the HMRU (n=16), or at an appropriate alternate location if subject travel to Ithaca was not possible (n=4). Subjects were eligible to participate between 7.0 and 7.7 y of age. Descriptive characteristics of the participants were obtained via parent report at the 7-y follow-up; these included child age, sex, visual acuity, red-green color vision deficiencies, grade in school, and race/ethnicity, and maternal education level and age. Additionally, potential bias from loss to follow-up was determined based on characteristics of the mothers assessed during the original feeding study, including maternal race/ethnicity, education level, and age.

Cognitive Measures

Visuospatial short-term memory. Children performed a challenging color-location memory task requiring them to recall the precise spatial locations of colored dots placed on a cartoon figure named Mr. Peanut^{26,27}. This task was selected because accurate recall required the binding of multiple features (i.e., unique color and location of each dot) in visual memory, a function in which the hippocampus has been shown to play a critical role when the bindings are complex, even at very brief delays^{28–32}. As previously discussed, improvements in offspring spatial cognition following maternal choline supplementation in rodents are likely due to improved functioning of the hippocampus¹.

The task was administered on a 30.48 cm x 19.05 cm Dell laptop using Inquisit 5 (Millisecond Software, Seattle, WA). Procedures for the present,

computerized version of this task were adapted from the original paper version and are summarized in **Figure 3.1**^{26,27}. Briefly, during each trial, the child was presented with an image of Mr. Peanut, showing one to five dots at 10 possible locations on the cartoon figure. The duration of this presentation phase corresponded to the number of dots on Mr. Peanut (i.e., 1 s for one dot trials; 2 s for two dot trials; etc.). After the presentation phase, a retention interval (RI) occurred in which a blank white screen was displayed. Following the RI, a blank version of Mr. Peanut appeared together with a column of five colored dots for the recall phase. The child was instructed to select the dots that had appeared in the preceding presentation phase and place them in the appropriate locations using a computer mouse.

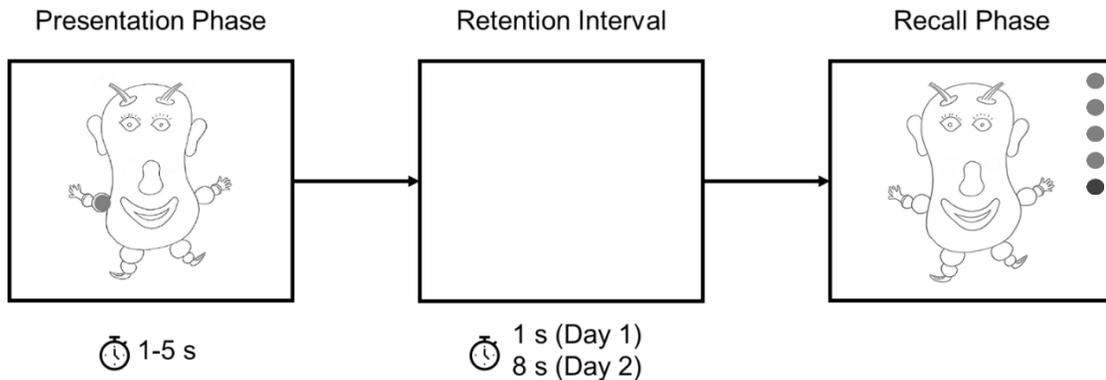


Figure 3.1 Mr. Peanut trial task diagram. Each trial began with the appearance of the cartoon figure on the laptop screen (presentation phase). The figure was presented for a duration (in seconds) equal to the number of dots on the figure (one to five). Following the presentation phase, a retention interval (RI) occurred in which a blank white screen was shown on the laptop. After the RI, a blank version of the cartoon figure appeared together with a column of five colored dots (recall phase). During the recall phase, the child was instructed to select the appropriate dot(s) from the column and place each dot in the precise location it had appeared in the preceding presentation phase using a computer mouse. The task began with four, one dot trials and advanced in difficulty level (number of dots to be remembered) if the child successfully completed at least one of the four trials at the difficulty level; the maximum difficulty level was five dots. The task was discontinued after the child failed all four trials at a difficulty level or after attempting all 20 trials. The task was administered twice: on testing day one the RI was 1 s and on testing day two the RI was 8 s. To prevent rehearsal during the 8 s RI, children counted aloud with a researcher until the cartoon figure reappeared.

The task began with four, one dot trials and advanced to the next difficulty level (number of dots to be remembered) if the child successfully completed at least one of the four trials in the level; the maximum difficulty level was five dots. The task was administered twice: on testing day one the RI was 1 s and on testing day two the RI was 8 s. To prevent rehearsal during the 8 s RI, children counted aloud until Mr. Peanut reappeared. Finally, to ensure that all children understood task rules and procedures, each child was required to successfully complete three practice trials on each day of testing before proceeding to the task.

Outcomes for the Mr. Peanut task included the maximum number of dots

recalled and the total number of trials correct. The maximum number of dots recalled is a proxy for visuospatial short-term memory capacity, the amount of information that can be encoded, maintained, and recalled after a brief delay. The total number of trials correct also reflects visuospatial short-term memory capacity but would be more sensitive to variations in performance consistency such as could be caused by inattentiveness or behavioral self-control problems.

Intelligence. To broadly characterize the cohort's cognitive functioning and intelligence, eight subtests of the Wechsler Preschool and Primary Scale of Intelligence, Fourth Edition (WPPSI-IV)³³ were administered. From these subtests, Full Scale Intelligence Quotient (IQ) and Primary Index Scale scores for Processing Speed (PSI), Verbal Comprehension (VCI), and Working Memory (WMI) were calculated. One child's primary language was not English and thus did not contribute data for Full Scale IQ and the VCI. It was not expected that Full Scale IQ or the VCI would be highly sensitive to the effects of maternal choline supplementation, however, previous research suggested that differences between treatment groups would be more likely to emerge for the PSI and the WMI. Because maternal choline supplementation in rodents has shown consistent benefits for offspring spatial memory^{1,2}, separate analyses of the two subtests which comprise the WMI (Picture Memory, a test of recognition memory; Zoo Locations, a test of object-location memory) were also conducted.

Video Coding

Video recordings of the Mr. Peanut task were reviewed using Behavioral Observation Research Interactive Software (BORIS, version 4.1.4)³⁴ to identify

instances of off-screen looking during the presentation phase which could potentially prevent full processing of the presented information. Each video was independently reviewed by two, trained study personnel who coded the start and stop times for each instance of off-screen looking. Discrepancies between coders were reviewed and resolved by a third, independent member of the research team.

Statistical Analysis

Descriptive characteristics of the final analytic sample were compared between treatment groups using Student's *t* tests for continuous variables and chi-square tests for categorical variables. Comparisons between the final analytic sample and subjects lost to follow-up or excluded from analysis were made in the same manner.

Data from the Mr. Peanut task were summarized by RI for each participant. Linear mixed models were used to assess the effect of third trimester choline intake on task performance with subject as a random effect. Child sex was included *a priori* as a fixed effect^{35,36} because research suggests that spatial memory varies by sex³⁷⁻⁴⁰. All models included the two-way interaction between treatment group (480 or 930 mg choline/d) and RI (1 or 8 s). To determine the extent to which the two outcome measures, maximum number of dots recalled and total number of trials correct, were independent from one another, Pearson's correlation coefficients were calculated for performance at the 1 s and 8 s RI.

Based on coding of the video recordings, the number of trials with and without off-screen looking were calculated by choline treatment group and

analyzed with a chi-square test of independence. The outcome of trials with and without off-screen looking were also compared using a chi-square test of independence. Finally, whether the outcome of trials with off-screen looking varied by choline treatment group was assessed using Fisher's Exact Test.

Data from the WPPSI-IV were analyzed using a general linear model and were adjusted *a priori* for child sex and maternal education (years of schooling). All statistical tests were two-sided and main effects were considered statistically significant at $p\text{-value} < 0.05$. Contrasts comparing the performance of treatment groups at each RI for the Mr. Peanut task were conducted for all outcomes, adjusted for multiple comparisons using a Bonferroni correction, and considered statistically significant at $p\text{-value} < 0.05$. All data were analyzed using SAS version 9.4 (SAS Institute Inc., Cary, NC).

Ethical Approval

Ethical approval for the 7-year follow-up and the original maternal choline feeding study were obtained from the Institutional Review Board for Human Participants at Cornell University. Written child assent and parental consent was obtained from all participants.

3.4 Results

Subject Characteristics

Twenty of the 26 children whose mothers completed the randomized controlled choline feeding trial returned for the 7-year cognitive follow-up and were successfully tested (77% retention; see **Figure 3.2**). One additional participant was re-recruited but did not comply with the testing protocol and their data were

excluded from analyses. The decision to exclude this participant was made before analysis commenced and before the data analyst was unblinded to the participant's group membership. Children lost to follow-up or excluded from analyses did not differ significantly from children included in analyses on a variety of demographic factors including child sex and maternal age, race, and education level (all $p \geq 0.27$; data not shown).

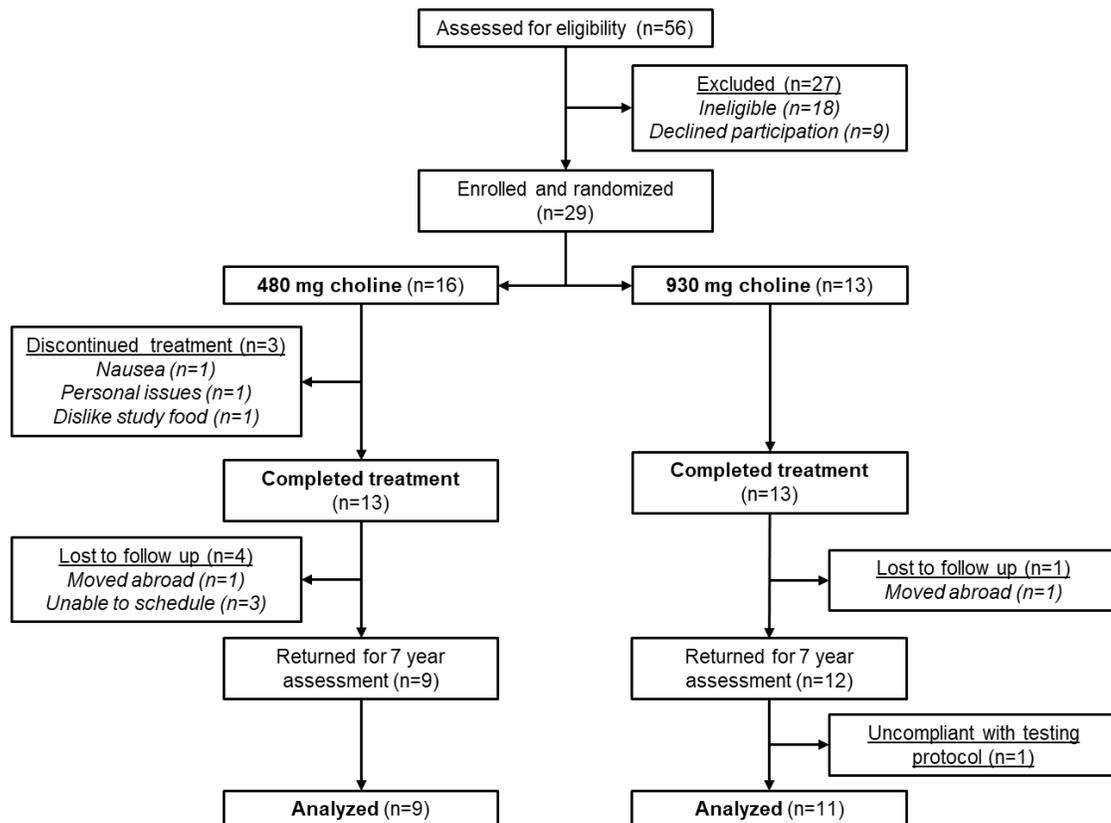


Figure 3.2 Participant flowchart for the assessment of the effect of 3rd trimester choline supplementation on child color-location memory task and WPPSI-IV performance at age 7 y

Characteristics of the participants and their mothers are presented in **Table 3.1**. Children in this sample were predominantly male and non-Hispanic

white. At the time of testing, all children had completed at least one year of formal schooling. Mothers of the children were generally highly educated, with 80% of women having received a bachelor's degree or higher. Treatment groups did not significantly differ on any maternal or child characteristic assessed, but children in the 480 mg/d group tended to have mothers with a higher level of educational attainment compared to children in the 930 mg/d group.

Table 3.1 Select demographic characteristics of participants in the final analytic sample and their mothers by 3rd trimester choline intake^a

	3 rd trimester choline intake		p ^b
	480 mg/d (n=9)	930 mg/d (n=11)	
Child characteristics			
Male	6 (67)	8 (73)	0.77
Age, mean y (SEM)	7.2 (0.2)	7.3 (0.2)	0.68
Highest grade completed			0.44
<i>Kindergarten</i>	3 (33)	2 (18)	
<i>First Grade</i>	6 (67)	9 (82)	
Normal or corrected to normal vision	9 (100)	11 (100)	NA
Red-green color vision deficiency	1 (11)	0 (0)	NA
Race			0.53
<i>Asian</i>	1 (11)	2 (18)	
<i>Black</i>	0 (0)	1 (9)	
<i>Native American</i>	0 (0)	1 (9)	
<i>White</i>	8 (89)	7 (64)	
Hispanic	2 (22)	2 (18)	0.82
Maternal characteristics			
Education level, mean y (SEM)	17.6 (0.4)	15.8 (0.7)	0.06
Age at child's conception, mean y (SEM)	28.4 (3.0)	27.6 (3.7)	0.61

^aAll values n (%) unless otherwise noted ^bReported p-values from Student's *t* tests (continuous variables) and chi-square tests (categorical variables)

Color-Location Memory

Off-screen looking: Off-screen looking during the presentation phase occurred in only 46 of the 488 trials administered (9.4% of trials). Trials with off-screen looking were failed at a higher rate than trials with no off-screen looking (78.3% v. 55.7%, $X^2 = 8.73$, $p < 0.005$). However, the percent of trials with off-screen looking ($X^2 = 0.64$, $p = 0.43$) and the percent failed when off-screen looking occurred (Fisher Exact Test: $p = 0.31$) did not vary by treatment group.

Maximum number of dots recalled: The maximum number of dots recalled was greater for children whose mothers consumed 930 mg choline/d (LS means [SEM]: 2.49 [0.23]) than for children whose mothers consumed 480 mg choline/d (LS means [SEM]: 1.81 [0.24]; $F_{(1,17)} = 4.53$, 95% CI [0.01, 1.34], $p = 0.048$; see **Figure 3.3, A**). A significant main effect of RI revealed that the maximum number of dots recalled was greater for the 1 s RI (LS means [SEM]: 2.66 [0.21]) compared to the 8 s RI (LS means [SEM]: 1.64 [0.21]; $F_{(1,18)} = 18.98$, 95% CI [0.53, 1.52], $p < 0.001$). Although no statistically significant interaction between choline group and RI was detected ($F_{(1,18)} = 1.11$, $p = 0.31$), contrasts at each RI suggested that the significant main effect of treatment group was due primarily to differential performance at the 1 s RI. Specifically, children in the 930 mg/d (v. 480 mg/d) group tended to pass more levels at the 1 s RI (LS means [SEM], 940 mg/d v. 480 mg/d: 3.12 (0.28) v. 2.20 (0.30); $t_{(31.8)} = 2.34$, adjusted 95% CI [-0.01, 1.85], adjusted $p = 0.05$) but not at the 8 s RI (LS means [SEM], 940 mg/d v. 480 mg/d: 1.85 (0.28) v. 1.42 (0.30); $t_{(31.8)} = 1.08$, adjusted 95% CI [-0.50, 1.35], adjusted $p = 0.58$; see **Figure 3.3, B**).

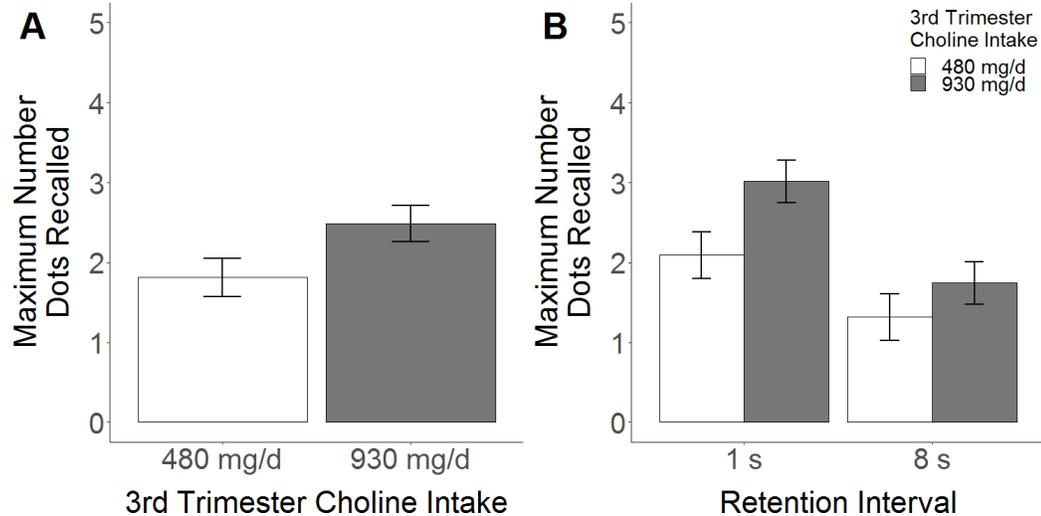


Figure 3.3 Maximum number of dots recalled. **(A)** Overall performance varied significantly by 3rd trimester choline intake ($p = 0.048$). **(B)** Although the interaction was not significant ($p = 0.31$), contrasts at each RI showed that treatment groups tended to differ at 1 s (adjusted $p = 0.05$) but not at 8 s ($p = 0.58$). Values represent LS means \pm SEM. 480 mg/d: $n = 9$; 930 mg/d: $n = 11$.

Total number of trials correct: The total number of trials correct did not differ between the 930 mg/d group (LS means [SEM]: 5.97 [0.66]) and the 480 mg/d group (LS means [SEM]: 4.50 [0.71]; $F_{(1,17)} = 2.52$, 95% CI [-0.49, 3.43], $p = 0.13$; see **Figure 3.4, A**). As with the maximum number of dots recalled, a significant main effect of RI was detected such that the total number of trials correct was greater at the 1 s RI (LS means [SEM]: 7.02 [0.59]) compared to the 8 s RI (LS means [SEM]: 4.50 [0.59]; $F_{(1,18)} = 34.40$, 95% CI [2.29, 4.85], $p < 0.0001$). The interaction between treatment group and RI was not statistically significant ($F_{(1,18)} = 1.70$, $p = 0.21$). As shown in **Figure 3.4, B**, similar to the pattern of results for the maximum number of dots recalled, contrasts at each RI showed that the 930 mg/d group tended to answer more trials correctly than the 480 mg/d group at the 1 s RI (LS means [SEM], 940 mg/d v. 480 mg/d: 8.16 [0.78] v. 5.89 [0.84];

$t_{(29.62)} = 2.04$, adjusted 95% CI [-0.36, 4.88], adjusted $p = 0.10$) but not at the 8 s RI (LS means [SEM], 940 mg/d v. 480 mg/d: 3.79 [0.78] v. 3.11 [0.84]; $t_{(29.62)} = 0.61$, adjusted 95% CI [-1.94, 3.30], adjusted $p > 0.99$). However, the total number of trials correct and maximum number of dots recalled were highly correlated at both the 1 s RI ($r = 0.92$, $p < 0.0001$) and the 8 s RI ($r = 0.90$, $p < 0.0001$), suggesting that these outcomes were not independent from one another.

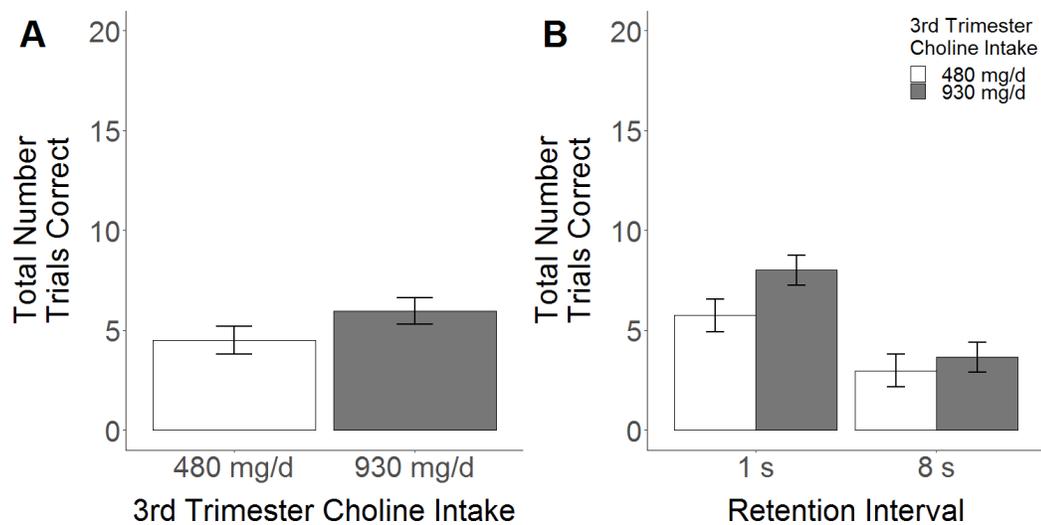


Figure 3.4 Total number of trials correct. **(A)** Overall performance did not vary by 3rd trimester choline intake ($p = 0.13$). **(B)** Contrasts at each RI showed that treatment groups tended to differ at the 1 s RI (adjusted $p = 0.10$) but not at the 8 s RI (adjusted $p > 0.99$), although the overall interaction was non-significant ($p = 0.21$). Values represent LS means \pm SEM. 480 mg/d: $n = 9$; 930 mg/d: $n = 11$.

Red-green color vision deficiency: One parent reported in the health history questionnaire that their child had a red-green color vision deficiency, a condition which may affect Mr. Peanut task scores because performance depends on the ability to discriminate between differently-colored dots. Although the child scored at the mean of their group (480 mg/d) for both the maximum number of

dots recalled and total number of trials correct, dot discrimination might have been more challenging for this child than for other children. In secondary analyses that excluded data from this participant, the results were essentially unchanged (see **Figure 3.5**).

Children in the 930 mg/d group (LS means [SEM]: 2.48 [0.23]) tended to pass more levels than children in the 480 mg/d group (LS means [SEM]: 1.82 [0.26]), but this effect was no longer statistically significant ($F_{(1,16)} = 3.89$, 95% CI [-0.05, 1.39], $p = 0.07$). Contrasts at each RI once again indicated that children in the 930 mg/d group tended to have a higher maximum number of dots recalled than children in the 480 mg/d group at the 1 s RI (LS means [SEM], 940 mg/d v. 480 mg/d: 3.12 [0.28] v. 2.19 [0.32]; $t_{(29.85)} = 2.21$, adjusted 95% CI [-0.06, 1.92], adjusted $p = 0.07$) but not the 8 s RI (LS means [SEM], 940 mg/d v. 480 mg/d: 1.85 [0.28] v. 1.44 [0.32]; $t_{(29.85)} = 0.97$, adjusted 95% CI [-0.59, 1.40], adjusted $p = 0.68$). A main effect of treatment group was not detected for the total number of trials correct (LS means [SEM], 940 mg/d v. 480 mg/d: 5.95 [0.68] v. 4.64 [0.75]; $F_{(1,16)} = 1.80$, 95% CI [-0.76, 3.39], $p = 0.20$). However, contrasts at each RI showed that children in the 930 mg/d group tended to pass a greater number of trials correct than children in the 480 mg/d group at the 1 s RI (LS means [SEM], 940 mg/d v. 480 mg/d: 8.13 [0.79] v. 5.95 [0.90]; $t_{(27.87)} = 1.86$, adjusted 95% CI [-0.60, 4.96], adjusted $p = 0.14$) but not the 8 s RI (LS means [SEM], 940 mg/d v. 480 mg/d: 3.77 [0.79] v. 3.32 [0.90]; $t_{(27.87)} = 0.38$, adjusted 95% CI [-2.33, 3.23], adjusted $p > 0.99$).

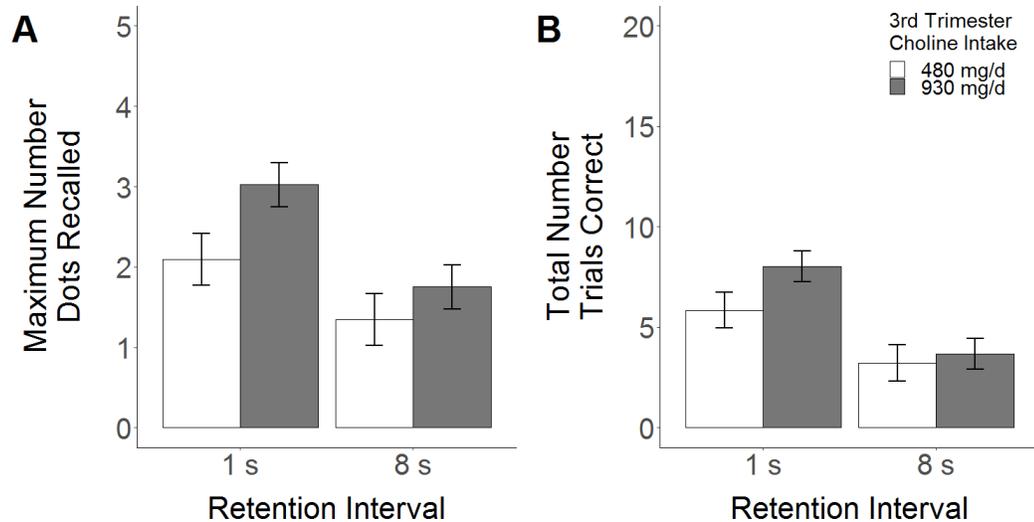


Figure 3.5 Color-location memory task performance excluding child with a red-green color vision deficiency. Exclusion of child with a red-green color vision deficiency did not alter the pattern of results for (A) maximum number of dots recalled or (B) total number of trials correct. Values represent least square means \pm SEM. 480 mg/d: $n = 8$; 930 mg/d: $n = 11$.

WPPSI-IV

Results from the WPPSI-IV are summarized in **Table 3.3**. Full Scale IQ, the primary index scale scores, and the two subtests which comprise the WMI (Picture Memory and Zoo Locations) did not vary by maternal choline intake at the $p < 0.05$ significance level. However, the pattern of results for the PSI, the WMI, and the Zoo Locations subtest were consistent with the hypothesized direction of effects. Because the Zoo Locations subtest and the total number of trials correct during the 1 s RI condition were expected to tap similar cognitive processes, the association between these two outcomes was assessed. Zoo Locations and the total number of trials correct were significantly correlated, indicating a moderate association between these outcomes ($r = 0.54$, $p = 0.01$).

Table 3.2 WPPSI-IV performance in all participants^a and by maternal choline intake during the third trimester^b

WPPSI-IV measure	All (n=20 ^c)	Third Trimester Choline Intake		p ^d
		480 mg/d (n=9)	930 mg/d (n=11 ^c)	
Full Scale IQ	110.2 (11.5)	108.0 (98.5, 117.5)	113.6 (105.3, 121.9)	0.35
PSI	107.8 (19.3)	104.6 (89.4, 119.7)	114.8 (102.6, 127.1)	0.26
VCI	110.0 (11.2)	113.2 (105.1, 121.4)	109.9 (102.7, 117.0)	0.51
WMI	101.0 (13.7)	97.0 (84.8, 109.2)	104.6 (94.8, 114.5)	0.31
<i>Picture Memory</i>	10.7 (2.6)	10.4 (8.1, 12.8)	11.2 (9.4, 13.1)	0.57
<i>Zoo Locations</i>	9.8 (2.9)	8.7 (6.1, 11.3)	10.5 (8.4, 12.6)	0.28

^aMean (SD); ^bLS Mean (95% Confidence Interval); ^cFull Scale IQ and VCI, missing = 1; ^dReported p-values from GLM, adjusted for maternal education level (years) and child sex. Abbreviations: PSI, Processing Speed Index; VCI, Verbal Comprehension Index; WMI, Working Memory Index.

3.5 Discussion

This study demonstrated that 7 y old children whose mothers consumed 930 mg choline/d during their 3rd trimester performed better on a demanding visuospatial short-term memory task than children whose mothers consumed 480 mg choline/d. Specifically, the maximum number of dots that could be recalled was greater for the 930 mg/d group than for the 480 mg/d group. Although the treatment group by RI interaction was not significant, contrasts at each RI showed that the 930 mg/d group tended to perform better at the 1 s RI, but not at the 8 s RI, relative to the 480 mg/d group. This pattern suggests that groups differed in their visuospatial short-term memory capacity. Similar results were observed for the total number of trials correct, with treatment groups tending to vary at the 1 s RI, but not the 8 s RI. Because these outcome measures were strongly correlated

($r \geq 0.90$), this suggests that group differences in visuospatial short-term memory capacity underlie both results.

Visuospatial short-term memory was also assessed by the Zoo Locations subtest of the WPPSI-IV which requires children to recall the location of animals on a grid following a brief delay. Notably, the results of the Zoo Locations subtest were consistent with the results for the Mr. Peanut task despite the lack of statistically significant group differences for this outcome. Specifically, performance on the two tasks were significantly correlated with one another, corroborating the inference that maternal choline supplementation improved visuospatial short-term memory capacity. It is possible that the Zoo Locations subtest showed less of an effect of prenatal choline supplementation than the Mr. Peanut task because it is a less challenging test of hippocampal function.

Rodent research suggests that beneficial effects of maternal choline supplementation on offspring spatial maze task performance may be due to changes in the hippocampus and/or septo-hippocampal cholinergic system^{8,10-14}, which plays important roles in spatial and explicit memory functions^{41,42}. Accordingly, we had predicted that treatment effects would be greater at the 8 s RI than at the 1 s RI due to the greater demands the former was expected to place on hippocampal function. However, this task was extremely challenging at both RIs, particularly at the 8 s RI due to the length of the delay and the prevention of rehearsal. The finding that group differences were apparently larger at the 1 s RI likely reflects the extreme difficulty of the 8 s RI condition and the consequent restricted range of outcome values. The tendency for the treatment groups to

differ at only the 1 s RI does not preclude improved functioning of the hippocampus as a source of group differences, however. Specifically, accurate recall for this task required successful complex feature binding in visual memory for which the hippocampus plays a critical role, even at brief retention intervals²⁸.

Although our findings are consistent with improved visuospatial short-term memory and functioning of the hippocampus, improvements to other cognitive processes and brain regions could also plausibly underlie observed group differences. In particular, selective attention plays an important role in facilitating visual memory throughout multiple stages of processing including the expectation, encoding, and maintenance of stimuli and their retrieval from memory^{43–45}. Consistent with this role of selective attention, the prefrontal cortex and parietal cortex have both been shown to support visuospatial short-term memory⁴⁶. Several reports have described beneficial effects of maternal choline supplementation on the functioning of the prefrontal cortex^{47–49}, although this has received considerably less attention than potential hippocampal mediation of visuospatial memory. Regardless of the cognitive process that underlies group differences in task performance, it manifested as superior visuospatial short-term memory capacity on this challenging color-location memory task.

Few studies in humans have assessed the relationship between prenatal choline intake and child memory. Broadly consistent with our findings, one observational study found a positive association between 2nd trimester choline intake and child visual memory at age 7 y¹⁸. In contrast, the only prior RCT which specifically assessed spatial memory did not detect a beneficial effect of maternal

choline supplementation on a test of short-term visuospatial memory²¹. However, in light of reported low infant compliance during testing and the collapsing of data across all levels of difficulty, it is unlikely that this latter study provided a sensitive assessment of group differences in memory function.

Full Scale IQ and other scales of the WPPSI-IV did not vary significantly by treatment group, although low statistical power likely precluded the detection of group differences. However, intriguingly, scales which measured aspects of cognition related to fluid intelligence⁵⁰ (i.e., the WMI and the PSI) tended to be higher in the 930 mg/d group than in the 480 mg/d group, whereas assessments which tapped aspects of crystallized intelligence (i.e., the VCI) did not. This is broadly consistent with prior research which generally has not detected benefits of higher prenatal choline exposure on child IQ¹⁶⁻¹⁸ but has detected benefits for aspects of cognition that may contribute to fluid intelligence¹⁸⁻²⁰.

This study has several strengths. First, maternal diet and supplement use during the study period was highly controlled, ensuring substantial group differences in choline intake. All women consumed the same menu of laboratory prepared food, and over 70% of the choline supplements were consumed under study personnel supervision. Second, the Mr. Peanut task was selected because it possessed characteristics likely to be sensitive to effects of the intervention on hippocampal-dependent memory function, the cognitive domain most consistently shown to be improved by maternal choline supplementation in rodents^{1,2}. This likely provided our study with a more sensitive assessment of the effects of maternal choline supplementation relative to studies which utilized only broad

measures of child development or intelligence. Third, all sessions were video recorded, providing a way to assess behavior during the Mr. Peanut task and helping to rule out alternative hypotheses for the observed pattern of results.

This study also has several limitations. First, the homogeneous composition of the participants (e.g., predominantly white, male, high level of maternal education) may limit the generalizability of our findings. Second, the small sample size of this follow-up increased the risk of chance findings⁵¹. However, the primary outcomes and analyses reported herein were prespecified, reducing our vulnerability to Type 1 errors. Finally, the Mr. Peanut task was not specifically designed to be equally distinguishable by individuals with color vision deficiencies. When data were analyzed excluding the child with a red-green color vision deficiency, the overall pattern of results did not change. However, it is possible that this child may have performed better had the task utilized an accessible color palette or alternative distinguishing feature, thereby reducing observed group differences. Importantly, this child performed at his group's average, indicating that his ability to distinguish colors was not completely impaired; it is unclear to what extent deficits in color discrimination affected his performance.

In conclusion, intake of 930 mg choline/d during the 3rd trimester of pregnancy improved child performance on a color-location memory task relative to 480 mg choline/d, indicative of superior visuospatial short-term memory capacity. In addition to this memory effect, beneficial effects of maternal choline supplementation on attentional control (**Chapter 2**) and planning (Nevins JEH et al., Manuscript in Preparation) have been detected in this cohort. This small, ancillary

follow-up provides preliminary, but compelling, evidence that maternal choline supplementation has beneficial effects on child cognition into the school-age years. Clinical trials specifically designed and powered to assess child cognitive and behavioral outcomes are needed to confirm and expand on these findings. If confirmed, it is of concern that most pregnant women in North America (approximately 90%) consume choline in quantities below the AI and most prenatal vitamins contain little or no choline⁵².

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**CHAPTER 4: EFFECT OF MATERNAL CHOLINE SUPPLEMENTATION
DURING LACTATION ON CHILD COGNITIVE OUTCOMES: AN ANCILLARY
FOLLOW-UP AT SEVEN YEARS OF AGE**

4.1 Abstract

Background: Rodent research demonstrates that perinatal maternal choline intake influences offspring cognitive functioning across the lifespan. However, little data exist on the functional effects of maternal choline intake, particularly during breastfeeding, on offspring cognition in humans. **Objective:** To assess the effect of maternal choline supplementation during breastfeeding on child cognitive functioning at age 7 y. **Design:** Women (approximately 5 weeks postpartum) intending to exclusively breastfeed were randomized to consume 480 mg or 930 mg choline/d for 10 weeks as part of a controlled-feeding study (n=28). An ancillary follow-up was conducted to assess cognition, including attention, spatial memory, and intelligence in their children at age 7 y (n=18). **Results:** Total choline metabolite concentration in breastmilk at the end of the intervention period did not vary by treatment group. Children in the 930 mg/d group tended to perform better on an attention test than children in the 480 mg/d group. In contrast, children in the 480 mg/d group performed better on a test of spatial memory than children in the 930 mg/d group. **Conclusions:** No consistent effect of varied maternal choline intake during lactation was detected in this cohort, with children in the 930 mg/d group tending to perform better on a test of attention and children in the 480 mg/d group performing better on a test of spatial memory. However, because total

choline metabolite concentrations in breastmilk at the end of the intervention period did not vary by treatment group, differences in performance cannot be attributed to differential infant choline intake from breastmilk, the mechanism by which the putative benefits of maternal choline supplementation would necessarily be mediated. It is possible that maternal choline supplementation during lactation may not have a beneficial effect on child cognition because breastmilk is naturally high in choline, but this study does not provide a strong test of this hypothesis.

4.2 Introduction

Choline is in high demand during pregnancy and lactation and plays important roles in child neurodevelopment as a precursor for the neurotransmitter acetylcholine, the methyl-donor betaine, and the phospholipids phosphatidylcholine (PC) and sphingomyelin. Thirty years of rodent research has demonstrated that perinatal maternal choline intake has life-long effects on offspring cognitive and behavioral functioning, with deprivation having adverse effects and supplementation having beneficial effects^{1,2}. Although most research has focused on the effects of varying prenatal choline intake, some studies have specifically assessed the effect of postnatal choline supplementation on offspring cognitive functioning. These studies detected beneficial effects, but the benefits were substantially less than those reported for prenatal supplementation^{3,4}. Furthermore, in rodent models of prenatal insults to brain development, such as fetal alcohol spectrum disorders and iron deficiency, postnatal choline supplementation has

been shown to attenuate the cognitive impairment^{5–10}. However, all postnatal choline supplementation studies provided the choline directly to offspring. No rodent studies have specifically assessed whether maternal choline supplementation during lactation has beneficial effects on offspring cognition.

Breastmilk is rich in choline, but concentrations are highly variable between women^{11–16}. Research in rodents^{17,18} and humans^{11–13} suggests that some of this variability can be explained by maternal choline intake and plasma/serum levels. Two randomized controlled trials (RCTs) found that concentrations of several choline metabolites were higher in women who consumed a choline supplement during pregnancy and/or lactation^{12,13}. One of these RCTs also found that postnatal choline supplementation increased phosphatidylethanolamine N-methyltransferase (PEMT) pathway enrichment of breastmilk PC¹². Compared to the cytidine diphosphate-choline pathway, the PEMT pathway produces PC molecules that are enriched in long-chain polyunsaturated fatty acids such as docosahexaenoic acid (DHA)¹⁶, important for infant brain development^{19–21}.

Only one study in humans has assessed whether the choline content of breastmilk is associated with infant cognition²². In this observational study, the free choline concentration of breastmilk was positively associated with infant recognition memory, assessed via electrophysiological measures, when levels of lutein or docosahexaenoic acid (DHA) in breastmilk were also high²². No published RCTs have assessed the exclusive effect of postpartum maternal choline supplementation on child cognition in women intending to breastfeed. To address the paucity of research in this area, the present study capitalized on an existing

controlled feeding study in which women intending to exclusively breastfeed were randomized to one of two levels of choline intake for 10 weeks during lactation. We followed-up the children of these women at 7 years of age, evaluating a wide range of cognitive and behavioral functions. The present report describes the results for tests of attention, spatial memory, and intelligence.

4.3 Methods

Ethical Approval

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

Study Design & Participants:

The present study is an ancillary 7-year follow-up of the children born to women who participated in a randomized, double-blind, parallel-group controlled choline feeding study early in the postpartum period during lactation. Details of the parent study (NCT01127022), including study diet, have been described elsewhere^{23,24}. Briefly, in 2009 – 2010, healthy, lactating women (approximately five weeks postpartum) aged ≥ 21 years were recruited to participate in a controlled choline feeding study from the Ithaca, NY region. Study eligibility was contingent on a variety of factors including the intention to exclusively breastfeed for the duration of the study, a willingness to comply with the study protocol, and general good health as assessed by a medical history questionnaire, complete blood

count, and blood chemistry profile. Exclusion criteria included the use of alcohol or tobacco products, a history of chronic disease, abnormal kidney or liver function, and the use of medications known to affect liver function.

Upon enrollment, women were randomized to consume either 480 mg or 930 mg choline/d for 10 weeks (approximately postpartum weeks 5 – 15). Target doses were achieved through the study diet (380 mg choline/d) and an additional choline supplement (100 mg or 550 mg choline/d). Study personnel prepared the choline supplement (choline chloride, Balchem Corp.) mixed with juice and provided it to participants in color-coded tubes so that participants and investigators were blinded to dose. For at least three days out of each week, women consumed at least one meal and their choline supplement in the Human Metabolic Research Unit at Cornell University under study personnel supervision. All other meals and supplements were provided as takeaways and consumed off site. Compliance to the study diet and choline supplement were high based on in-lab monitoring and return of supplement and food containers. In addition to the study diet and choline supplement, women consumed a daily choline-free prenatal multivitamin (Pregnancy Plus, Fairhaven Health LLC), a daily 200 mg DHA supplement daily (Neuromins, Nature's Way Products), and a thrice weekly 250 mg potassium and 250 mg magnesium supplement (General Nutrition Corp.).

In 2016 – 2018, children born to women who participated in the controlled feeding study were invited to participate in an ancillary follow-up to assess the effect of maternal choline supplementation during breastfeeding on child cognition. Children were tested between 7.0 and 7.7 years of age. Demographic

information on the children and their mothers were obtained via parent report at the 7-year follow-up and included child sex, race, ethnicity, age, grade in school, visual acuity, and red-green colorblindness, and maternal age and education level. Characteristics of the mothers collected at the time of the original feeding study, including maternal age, race, ethnicity, education level, and breastmilk choline content at study entry and end, were used to assess bias from loss to follow-up. Methods used to quantify breastmilk choline metabolites were described previously¹².

Cognitive Measures

Follow-up assessments were conducted at Cornell University by trained study personnel blinded to group assignment and consisted of two, 90-minute testing sessions held on consecutive days. A variety of cognitive and behavioral functions were assessed during the testing sessions, including attention, spatial memory, and IQ. Computerized tasks were administered on a 30.48 cm x 19.05 cm Dell laptop.

Attention. Children were administered a 216 trial, 11.5-minute computerized signal detection task (referred to as the “Sustained Attention Task [SAT]”)²⁵, programmed in OpenSesame Version 3.0.4²⁶. Selection of the SAT was based on evidence that offspring of choline supplemented mice exhibited superior performance on a rodent analog of the task²⁷. Briefly, children were instructed to watch a gray screen and to indicate whether they saw or did not see a small (5 mm x 5 mm square), low-contrast signal of variable duration (17, 29, or 50 ms) during each task trial (see **Figure 4.1**).

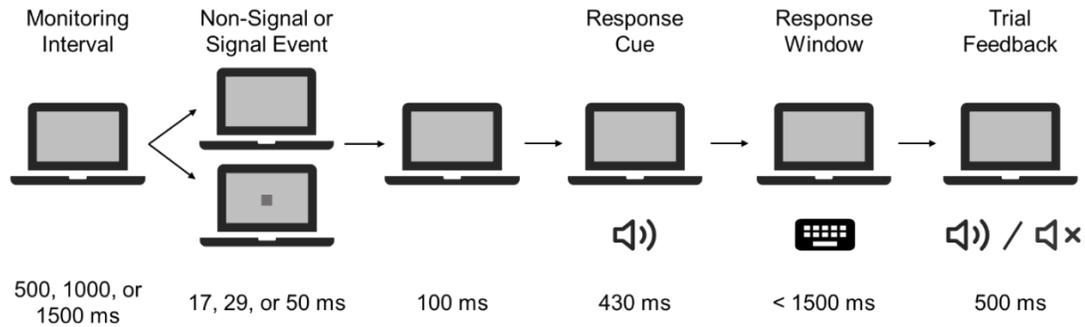


Figure 4.1. Sustained Attention Task (SAT).

Each trial began with a monitoring interval of variable duration (500, 1000, or 1500 ms) to prevent anticipatory responding. Following the monitoring interval, a signal or non-signal event occurred. Signals were presented randomly on 50% of trials, with an equal number of signal and non-signal events occurring every 18 trials. One hundred ms after the signal or non-signal event, a 430 ms auditory cue prompted children to indicate whether they saw or did not see a signal. Children had 1500 ms to respond by pressing the appropriate, pre-specified key on the testing laptop (determined by handedness). If a correct response was made, a 500 ms tone was played; no feedback was given for incorrect responses or omissions. To ensure understanding of task rules and procedures, each child was administered a minimum of 12 practice trials and was only allowed to proceed to the task after training to criterion.

The outcome of each SAT trial was categorized as a hit, miss, false alarm, correct rejection, or omission based on trial type and child response (see **Table 4.1**). Performance on the SAT was primarily assessed through a measure termed “SAT score” which integrates performance across both signal and non-signal trials and is calculated using the following formula, $(\% \text{ Hits} - \% \text{ False Alarms}) /$

$(2[\% \text{ Hits} + \% \text{ False Alarms}] - [\% \text{ Hits} + \% \text{ False Alarms}]^2)$, where $\% \text{ Hits} = \text{Hits} \div (\text{Hits} + \text{Misses})$ and $\% \text{ False Alarms} = \text{False Alarms} \div (\text{False Alarms} + \text{Correct Rejections})$ ²⁵. SAT score ranges from -1 to +1 with scores ≤ 0 indicating an inability to distinguish signal and non-signal trials, and a score of +1 representing perfect responding²⁵. The two components of SAT score (percent hits and percent false alarms) and omission errors were analyzed to gain insight into SAT score results and to assess task engagement and understanding, respectively.

Table 4.1 SAT trial outcomes defined by trial type and child response

Trial Type	Child Response		
	<i>Saw Signal</i>	<i>Did Not See Signal</i>	<i>No Response</i>
Signal Trial	Hit	Miss	Omission
Non-Signal Trial	False Alarm	Correct Rejection	

Visuospatial short-term memory. Children were administered a challenging color-location memory task which required them to recall the location of colored dots on a cartoon figure (“Mr. Peanut”; see **Figure 4.2**)^{28,29}. Because improved hippocampal functioning is hypothesized to underlie the beneficial effects of maternal choline supplementation on offspring spatial memory in rodents^{1,2}, this task was selected because performance was expected to depend heavily on hippocampal function. Specifically, accurate recall on this task requires the binding of multiple features (i.e., the exact and unique color and location of each dot) in visual-spatial memory. The hippocampus plays an important role in this function when the feature binding is complex, even at short retention intervals^{30–34}.

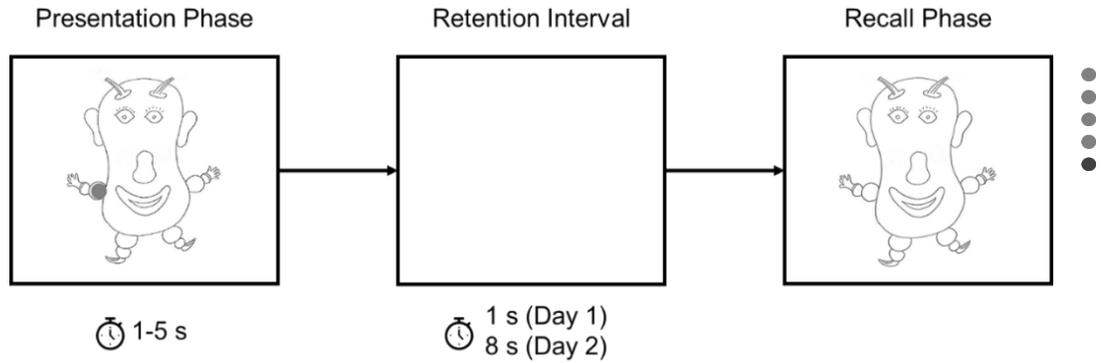


Figure 4.2 Mr. Peanut color-location memory task diagram.

Briefly, each task trial began with a presentation phase in which an image of a cartoon figure decorated with one to five dots, each of a unique color, was displayed on the computer screen. The duration of the presentation phase (in seconds) was equal to the number of dots on the figure (e.g., 1-dot trials had a 1 s presentation; 2-dot trials had a 2 s presentation; etc.). Following the presentation phase, a blank, white screen was displayed for the retention interval (RI). After the RI, an empty version of the cartoon figure reappeared, along with a column of five dots of unique colors, for the recall phase. During the recall phase, children attempted to place the dot(s) where they had been located on Mr. Peanut before the RI. Trials were administered in order of difficulty level, beginning with 1-dot trials and ending at a maximum of 5-dot trials. Each difficulty level consisted of four trials, and children were only administered the next difficulty level if they successfully completed at least one trial in the preceding level. The task was administered twice: once with a 1 s RI and once with an 8 s RI. Children counted aloud with the experimenter during the 8 s RI to prevent rehearsal.

To ensure children understood task rules and procedures, each child

needed to successfully complete three practice trials on each day of testing before proceeding to the task. Outcomes for the Mr. Peanut task included the maximum number of dots recalled (i.e., highest level passed), a proxy for visuospatial short-term memory capacity, and the total number of correct trials, which is affected by visuospatial short-term memory capacity and consistency of performance. The Mr. Peanut task was programmed and administered using Inquisit 5 (Millisecond Software, Seattle, WA, USA).

Dimensions of intelligence. The Wechsler Preschool and Primary Scale of Intelligence, Fourth Edition (WPPSI-IV)³⁵ was administered to characterize dimensions of intelligence and general cognitive functioning in the cohort. Eight subtests of the WPPSI-IV were administered and used to calculate full scale intelligence quotient (IQ) and three primary index scales (Processing Speed Index, PSI; Verbal Comprehension Index, VCI; Working Memory Index, WMI). Based on rodent research which shows a consistent benefit of maternal choline supplementation on offspring spatial memory, the two subtests which compose the WMI (Picture Memory, test of recognition memory; Zoo Locations, test of object-location spatial memory) were analyzed individually.

Video Coding

Video recordings of the testing sessions were reviewed using Behavioral Observation Research Interactive Software Version 4.1.4³⁶ to identify instances in which participants were looking off-screen during the SAT and during the presentation phase of the Mr. Peanut task. The primary motivation for coding these instances of off-screen looking was to assess whether they contributed to

the results for these two tasks. Instances of off-screen looking were coded independently by two, trained study personnel blinded to treatment group. When the coders were not in agreement, the instance of off-screen looking was reviewed, and consensus was reached through discussion.

Statistical Analysis

Characteristics of the children and their mothers were compared between treatment groups using Student's *t* tests for continuous variables and chi-square tests for categorical variables. Comparisons between subjects lost to follow-up and those included in the final analysis were made in the same manner.

SAT: Linear mixed models were used to assess the effect of postpartum maternal choline supplementation on SAT score, percent hits, and percent false alarms; a generalized linear mixed model with a binomial distribution was used to assess the effect of postpartum maternal choline intake on omissions. SAT performance was modeled as a function of task block (3 blocks of 72 trials) and signal duration (as applicable), with random effects of subject and block nested within subject. Child sex was included as a fixed effect, identified *a priori* as the sole covariate^{37,38} due to research which suggests that child performance on several tests of attention varies by sex³⁹⁻⁴³. Analyses for SAT score and percent hits included the three-way interaction and the corresponding two-way interactions between treatment group, signal duration, and task block. The analysis of omissions did not contain the three-way interaction as the model did not converge when this interaction term was included. The analysis of percent false alarms did not include the three-way interaction because this outcome only involves non-

signal trials; therefore, signal duration was not a relevant variable. Single degree of freedom linear interaction contrasts were constructed to compare change in performance across the task (block one to block three) as the primary test of the treatment group by task block interaction. Sensitivity analyses were conducted excluding trials missed due to off-screen looking to evaluate their influence on overall performance. Whether having an occurrence of off-screen looking varied by treatment group was assessed with Fisher's Exact Test. Finally, total time looking off-screen was compared between groups using a Wilcoxon rank sum test.

Mr. Peanut: Linear mixed models were used to assess the effect of postpartum maternal choline supplementation on the Mr. Peanut task. Performance was modeled as a function of RI, with subject as a random effect and child sex included *a priori* as a fixed effect^{37,38} due to research which suggests that spatial memory varies by sex⁴⁴⁻⁴⁷. All models included the two-way interaction between treatment group and RI. The number of trials with and without off-screen looking were calculated for each choline treatment group and compared with a chi-square test of independence. The outcome of trials (i.e., correct or incorrect) with and without off-screen looking were also compared using a chi-square test of independence. Finally, the outcome of trials with off-screen looking were calculated for each choline treatment group and compared using Fisher's Exact Test.

WPPSI-IV: A general linear model was used to assess data from the WPPSI-IV and models were adjusted *a priori* for maternal education (continuous) and child sex.

Statistical tests were two-sided and main effects, pairwise comparisons, and planned contrasts were considered statistically significant a p-value < 0.05. Pairwise comparisons were adjusted for multiplicity using a Bonferroni correction. All data were analyzed using SAS version 9.4 (SAS Institute Inc., Cary, NC).

4.4 Results

Loss to Follow-Up

Eighteen of the 28 children whose mothers completed the randomized controlled choline feeding study during lactation returned for the 7-year cognitive follow-up (64% retention; see **Figure 4.3**). Breastmilk choline measured at feeding study entry varied by follow-up status: children re-recruited had mothers with a higher total choline metabolite concentration in breastmilk at study entry (mean [SEM]: 1304.6 [58.9] $\mu\text{mol/l}$) compared to those lost to follow-up (mean [SEM]: 1079.4 [58.2] $\mu\text{mol/l}$; $t_{(26)} = 2.49$, 95% CI [39.2 $\mu\text{mol/l}$, 411.1 $\mu\text{mol/l}$], $p = 0.02$). Among those lost to follow-up, children whose mothers were randomized to consume 930 mg choline/d (mean [SEM]: 971.8 [70.9] $\mu\text{mol/l}$) tended to have a lower total choline metabolite concentration in breastmilk at baseline than children whose mothers were randomized to consume 480 mg choline/d (mean [SEM]: 1187.0 [66.7] $\mu\text{mol/l}$; $t_{(8)} = 2.21$, 95% CI [-439.6, 9.2], $p = 0.06$). Although not statistically significant, a higher percentage of males were lost to follow-up compared to females (46.2% v. 26.7%, Fisher's Exact Test: $p = 0.18$). No other statistically significant differences between children lost to follow-up and those successfully re-recruited were detected for a variety of other factors including

maternal education, race, ethnicity, and age, breastmilk total choline metabolite concentration at study end, weeks postpartum at start of the feeding study, and infant gestational age at birth (all $p \geq 0.38$; data not shown).

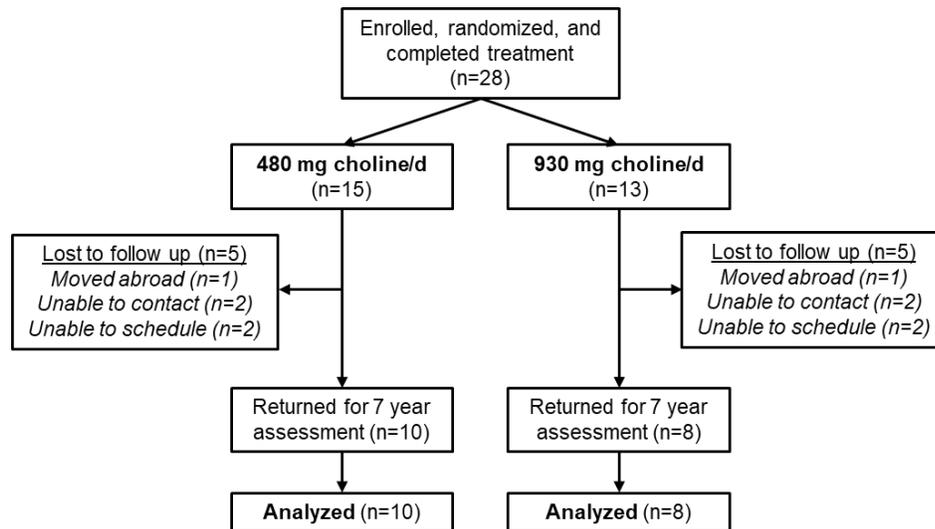


Figure 4.3 Participant flowchart for the assessment of the effect of maternal choline intake during lactation on child cognitive outcomes at age 7 y

Participant Characteristics

Characteristics of the participants and their mothers are presented in **Table 4.2**. The sample was predominantly non-Hispanic white, and all children had completed at least one year of formal schooling (i.e., Kindergarten). Mothers of the children were highly educated, the majority (72.2%) having earned a bachelor's degree or higher. There were no statistically significant differences between treatment groups on any maternal or child factors assessed, including total choline metabolite concentration in breastmilk at the end of the feeding study. However, the total choline metabolite concentration in breastmilk at study entry

tended to be lower in women randomized to the 930 mg choline/d group compared to women randomized to the 480 mg/d group

Table 4.2 Selected characteristics of children who participated in the 7-year follow-up and their mothers by choline treatment group^a

	Maternal postpartum choline intake		p ^b
	480 mg/d (n=10)	930 mg/d (n=8)	
Child characteristics			
Male	5 (50.0)	2 (25.0)	0.28
Age, mean y (SEM)	7.4 (0.1)	7.3 (0.1)	0.37
Highest grade completed			0.51
<i>Kindergarten</i>	2 (20.0)	3 (37.5)	
<i>First Grade</i>	7 (70.0)	5 (62.5)	
<i>Second Grade</i>	1 (10.0)	0 (0)	
Normal or corrected to normal vision	11 (100)	8 (100)	NA
Red-green color vision deficiency	0 (0)	0 (0)	NA
Race			0.41
<i>Biracial^c</i>	2 (20.0)	0 (0)	
<i>Black</i>	2 (20.0)	2 (25.0)	
<i>White</i>	6 (60.0)	6 (75.0)	
Hispanic	2 (20.0)	0 (0)	0.18
Maternal characteristics			
Study entry, mean wk postpartum (SEM)	4.4 (0.8)	5.0 (0.8)	0.57
Total choline ^d metabolites in breastmilk			
Study entry, mean $\mu\text{mol/l}$ (SEM)	1372.2 (86.7)	1220.1 (72.2)	0.21
Study end, mean $\mu\text{mol/l}$ (SEM)	1084.2 (84.1)	1129.9 (82.3)	0.71
Education level at follow-up, mean y (SEM)	15.8 (0.6)	15.8 (0.8)	0.96
Age at follow-up, mean y (SEM)	36.6 (1.8)	36.0 (1.1)	0.79

^aAll values n (%) unless otherwise noted, ^bReported p-values from Student's *t* test for continuous variables and from chi-square tests for categorical variables, ^cAsian/White: n=1; Black/White: n=1, ^dIncludes all measured choline-containing biomolecules including free choline, glycerophosphocholine, phosphocholine, phosphatidylcholine, and sphingomyelin

Attention

SAT score, task design effects: SAT score varied by signal duration ($F_{(2,96)} = 26.68$, $p < 0.0001$) such that performance on 17 ms trials was poorer than that on 29 ms ($t_{(96)} = 6.29$, adjusted 95% CI [-0.17, -0.08], adjusted $p < 0.001$) or 50 ms trials ($t_{(96)} = 6.36$, adjusted 95% CI [-0.18, -0.08], adjusted $p < 0.001$); performance did not differ between 29 ms and 50 ms trials ($t_{(96)} = 0.07$, adjusted 95% CI [-0.05, 0.05], adjusted $p > 0.99$). Although not statistically significant, SAT score also tended to vary by task block ($F_{(2,32)} = 2.96$, $p = 0.07$) such that performance tended to be worse at the third task block compared to the first task block ($t_{(32)} = 2.14$, adjusted 95% CI [-0.22, 0.02], adjusted $p = 0.12$) or the 2nd block ($t_{(32)} = 2.05$, adjusted 95% CI [-0.22, 0.02], adjusted $p = 0.14$); performance did not differ between the first and second task blocks ($t_{(32)} = 0.06$, adjusted 95% CI [-0.12, 0.12], adjusted $p > 0.99$).

SAT score, maternal choline effects: A main effect of treatment group was not detected for SAT score ($F_{(1,15)} = 0.46$, 95% CI [-0.15, 0.29], $p = 0.51$). However, a statistically significant interaction between signal duration and treatment group was detected ($F_{(2,96)} = 3.24$, $p = 0.04$). Contrasts at each signal duration revealed no statistically significant differences between treatment groups (17 ms: $t_{(16.52)} = 1.12$, 95% CI [-0.16, 0.40], adjusted $p = 0.84$; 29 ms: $t_{(16.52)} = 0.16$, 95% CI [-0.26, 0.30], adjusted $p > 0.99$; 50 ms: $t_{(16.52)} = 0.69$, 95% CI [-0.21, 0.36], adjusted $p > 0.99$; see **Figure 4.4, Panel A**). But, a *post-hoc* contrast revealed that the difference in SAT scores between treatments groups was greater for 17 ms trials than the average treatment group difference for 29 ms and 50 ms trials

($t_{(96)} = 2.12$, 95% CI [0.005, 0.14], $p = 0.04$). No additional two- or three-way interactions were detected (all adjusted $p \geq 0.27$; data not shown).

Percent hits, task design effects: Percent hits varied by signal duration ($F_{(2,96)} = 29.73$, $p < 0.0001$) such that performance was poorest for 17 ms trials relative to 29 ms trials ($t_{(96)} = 6.66$, adjusted 95% CI [-19%, -9%], adjusted $p < 0.001$) and 50 ms trials ($t_{(96)} = 6.70$, adjusted 95% CI [-19%, -9%], adjusted $p < 0.001$); performance for 29 ms and 50 ms trials did not differ from one another ($t_{(96)} = 0.04$, adjusted 95% CI [-5%, 5%], adjusted $p > 0.99$). Percent hits tended to vary by task block ($F_{(2,32)} = 2.95$, $p = 0.07$) such that percent hits tended to be lower during the third task block compared to the first task block ($t_{(96)} = 2.27$, adjusted 95% CI [-15%, 1%], adjusted $p = 0.09$); percent hits during second task block did not differ from either the third task block ($t_{(96)} = 1.88$, adjusted 95% CI [-2%, 13%], adjusted $p = 0.21$) or the first task block ($t_{(96)} = 0.39$, adjusted 95% [-6%, 9%], adjusted $p > 0.99$).

Percent hits, maternal choline effects: No main effect of treatment group was detected ($F_{(1,15)} = 0.49$, 95% CI [-7%, 14%], $p = 0.49$). A marginal interaction between treatment group and signal duration was detected ($F_{(2,96)} = 3.00$, $p = 0.054$). At no signal duration did the treatment groups differ from one another (17 ms: $t_{(23.26)} = 1.54$, adjusted 95% CI [-6%, 22%], adjusted $p = 0.41$; 29 ms: $t_{(23.26)} = 0.36$, adjusted 95% CI [-16%, 12%], adjusted $p > 0.99$; 50 ms: $t_{(23.26)} = 0.69$, adjusted 95% CI [-10%, 18%], adjusted $p > 0.99$; see **Figure 4.4, Panel B**). However, a *post-hoc* contrast revealed that the difference in percent hits between treatment groups was greater for 17 ms trials than the average treatment group

difference for 29 ms and 50 ms trials ($t_{(96)} = 2.04$, 95% CI [0.002, 0.15], $p = 0.04$). No additional statistically significant two- or three-way interactions were detected (all $p \geq 0.26$; data not shown).

Percent false alarms, task design effects: Performance did not vary by task block ($F_{(2,32)} = 2.00$, $p = 0.15$; block 1: 17.6% [3.3%], block 2: 16.9% [3.3%], block 3: 21.2% [3.3%], all mean [SEM]).

Percent false alarms, maternal choline effects: Performance on non-signal trials did not vary by treatment group ($F_{(1,15)} = 0.26$, 95% CI [-16%, 10%], $p = 0.62$; see **Figure 4.4, Panel C**). No interaction between treatment group and task block was detected ($t_{(32)} = 0.41$, 95% CI [-7%, 11%], $p = 0.68$).

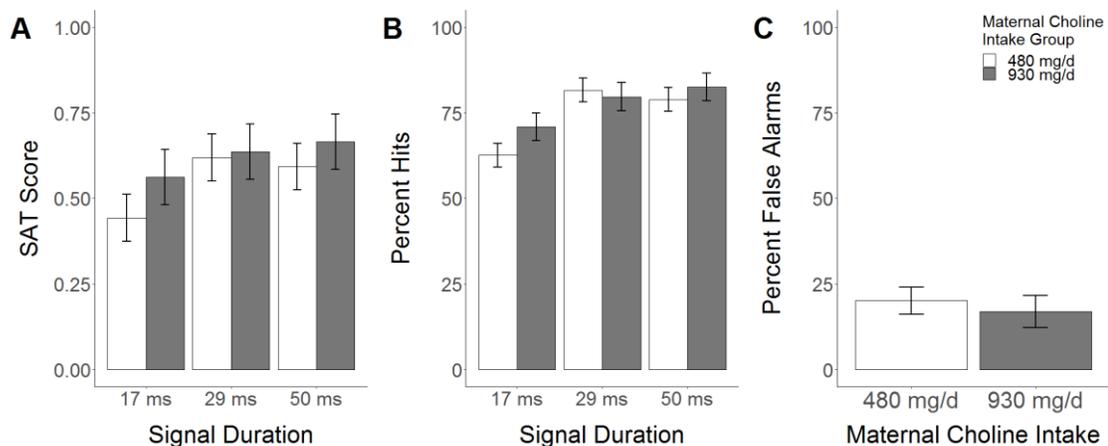


Figure 4.4 Performance on the SAT as a function of maternal choline intake and signal duration (as applicable) the following outcomes: **(A)** SAT score, **(B)** percent hits, **(C)** percent false alarms. No main effect of treatment group (all $p \geq 0.49$) was detected for any outcome. The interaction between treatment group and signal duration was statistically significant for SAT score ($p = 0.048$) and marginally significant for percent hits ($p = 0.05$). However, pairwise comparisons revealed no differences between treatment groups at any signal duration for both outcomes (all adjusted $p \geq 0.41$). Values represent least square (LS) means \pm SEM. 480 mg/d: $n = 10$; 930 mg/d: $n = 8$.

Omissions, task design effects: Omissions were rare (Median [IQR]: 5.1% [1.9%, 7.4%]), suggesting that children were engaged with the task and complied with task rules. Omissions varied by signal duration ($F_{(3,197)} = 10.66$, $p < 0.0001$) such that more omissions occurred on non-signal trials than on signal trials of any duration (all $t_{(197)} \geq 2.65$, adjusted $p \leq 0.05$). Omissions did not vary by task block ($F_{(2, 83.59)} = 1.00$, $p = 0.37$).

Omissions, maternal choline effects: Omissions did not vary by treatment group ($F_{(1,16.22)} = 0.01$, $p = 0.93$). No statistically significant interactions between treatment group and signal duration or task block were detected (all $p \geq 0.82$; data not shown).

Off-screen looking. Review of SAT video recordings revealed that only 0.06% of trials (25 out of 3888) were missed due to children looking off-screen. Eleven children out of 18 looked off-screen at least once; the percent of children who looked off-screen did not vary between groups (Fisher's exact test: $p > 0.99$). Overall, the amount of time spent looking off-screen was minimal (Median [IQR] 0 s [0 s – 2.6 s]) and did not vary by treatment groups ($Z = 0.60$; $p = 0.55$). Sensitivity analyses which excluded trials in which children were off-screen did not yield results which altered the interpretation of the initial analyses (see **Appendix E**).

Color-Location Memory

Off-screen looking. Off-screen looking during the presentation phase occurred in 56 of the 496 trials administered. Children in the 480 mg/d group tended to have a higher percent of trials with off-screen looking than the 930 mg/d group

(13.4% v. 8.3%; $X^2 = 3.0$, $p = 0.08$). Trials in which off-screen looking occurred were more likely to be failed than trials in which no off-screen looking occurred (83.9% v. 56.6%, $X^2 = 15.4$, $p < 0.001$). However, the percent of trials with off-screen looking that were failed did not vary by treatment group (Fisher Exact Test: $p > 0.99$).

Maximum number of dots recalled. The maximum number of dots recalled varied by RI ($F_{(1,16)} = 18.25$, 95% CI [0.46, 1.37], $p < 0.001$) such that more levels were passed at the 1 s RI compared to the 8 s RI. The maximum number of dots recalled also varied by treatment group ($F_{(1,15)} = 4.62$, 95% CI [-1.00, -0.004], $p = 0.048$) such that children in the 480 mg/d group performed better than the 930 mg/d group. The interaction between treatment group and RI was not statistically significant ($F_{(1,16)} = 0.99$, $p = 0.33$). However, post-hoc pairwise comparisons indicate that treatment groups did not differ significantly at the 1 s RI ($t_{(30.55)} = 0.91$, adjusted 95% CI [-1.03, 0.46], adjusted $p = 0.74$) but tended to differ at the 8 s RI ($t_{(30.55)} = 2.26$, adjusted 95% CI [-1.46, 0.03], adjusted $p = 0.06$), consistent with the visual pattern of results (see **Figure 4.5, Panel A**).

Total number of trials correct. A significant main effect of RI on the total number of trials correct was detected ($F_{(1,16)} = 241.2$, 95% CI [3.41, 4.49], $p < 0.0001$) such that more trials were passed at the 1 s RI relative to the 8 s RI. No main effect of treatment group on trials correct was detected ($F_{(1,15)} = 1.08$, 95% CI [-2.70, 0.93], $p = 0.31$) but a statistically significant interaction between treatment group and RI was ($F_{(1,16)} = 4.68$, $p = 0.046$). Visually, differences between the treatment groups appeared to emerge at the 8 s RI. However, treatment

groups did not significantly differ at either the 1 s RI ($t_{(17.68)} = 0.38$, adjusted 95% CI [-2.51, 1.84], adjusted $p > 0.99$) or the 8 s RI ($t_{(17.68)} = 1.62$, adjusted 95% CI [-3.61, 0.74], adjusted $p = 0.24$) (see **Figure 4.5, Panel B**).

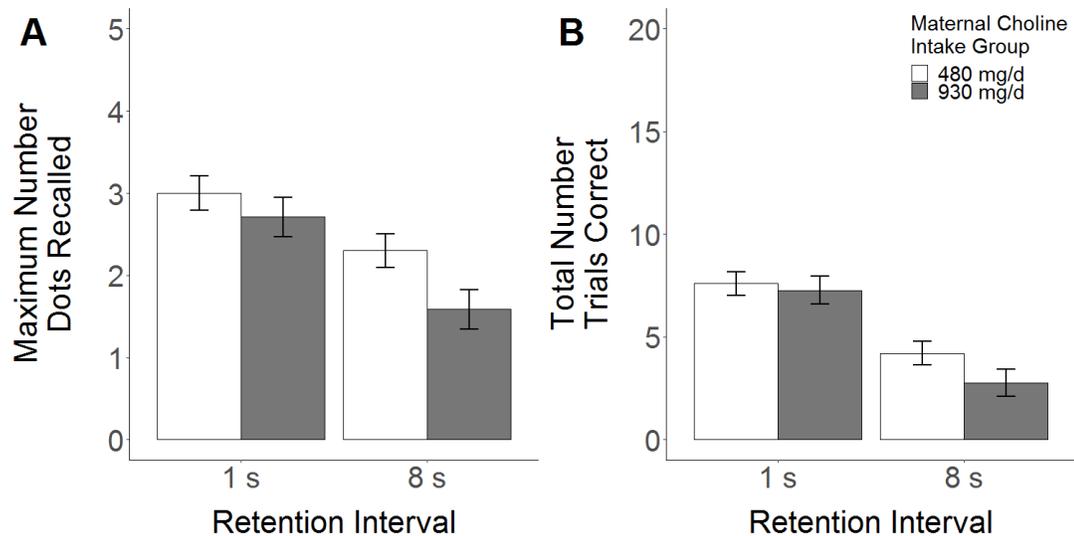


Figure 4.5 Color-location memory task performance as a function of retention interval and maternal choline intake for (A) the maximum number of dots recalled and (B) the total number of trials correct. Performance varied significantly by RI for each measure ($p < 0.0001$). The maximum number of dots recalled ($p = 0.048$), but not the total number of trials correct ($p = 0.31$), was significantly greater for the 480 mg/d group compared to the 930 mg/d group. The treatment group by RI interaction for number of trials correct was statistically significant ($p = 0.046$), however, performance did not vary by treatment group at either the 1 s or 8 s RI (both adjusted $p \geq 0.24$). Values represent LS means \pm SEM. 480 mg/d: $n = 10$; 930 mg/d: $n = 8$.

Dimensions of Intelligence

Treatment groups did not differ on any measure of performance on the WPPSI-IV including Full Scale IQ, any primary index scale, and the two subtests which comprise the Working Memory Index (see **Table 4.3**).

Table 4.3 WPPSI-IV performance in all participants^a and by postpartum maternal choline intake^b

WPPSI-IV measure	All (n=18)	Postpartum maternal choline intake		p ^c
		480 mg/d (n=10)	930 mg/d (n=8)	
Full Scale IQ	114.1 (12.8)	115.1 (107.2, 123.1)	113.2 (103.8, 122.7)	0.75
PSI	109.3 (17.0)	113.2 (101.4, 125.1)	103.1 (88.9, 117.3)	0.26
VCI	112.3 (11.8)	109.0 (102.9, 115.1)	116.0 (108.7, 123.3)	0.14
WMI	104.8 (11.6)	104.9 (96.5, 113.2)	104.7 (94.7, 114.7)	0.98
<i>Picture Memory</i>	11.5 (2.4)	11.4 (9.7, 13.1)	11.4 (9.3, 13.4)	0.98
<i>Zoo Locations</i>	10.1 (2.3)	10.1 (8.5, 11.7)	10.3 (8.3, 12.2)	0.89

^aValues reported as mean (SD); ^bValues reported as LS means (95% CI); ^cReported p-values from GLM, adjusted for child sex and maternal education level (years)

4.5 Discussion

This ancillary follow-up of a randomized controlled feeding trial was conducted to evaluate the effects of maternal choline supplementation during breastfeeding on child cognition. Visual inspection of the pattern of findings for the SAT, coupled with the statistically significant *post-hoc* contrasts demonstrating that differences between treatment groups were greatest for trials with the briefest cues, suggest that children in the 930 mg/d group may have exhibited superior performance on this task. Contrary to predictions, the 930 mg/d group performed significantly less well in the color-location memory task than the 480 mg/d group. No statistically significant differences between treatment groups were detected on the WPPSI-IV which assessed dimensions of intelligence.

Prior to drawing conclusions from these results, it is necessary to examine whether substantive differences in breastmilk choline content were achieved by the intervention, the mechanism by which the putative cognitive benefits would

be mediated. Critically, total choline concentrations in breastmilk did not vary by treatment group in the subset of women whose children participated in the cognitive follow-up at 7 years of age. This suggests that infant choline intake during the study period may not have varied meaningfully by treatment group in those assessed for this ancillary follow-up. It is therefore most likely that the observed differences in cognitive performance are not attributable to postnatal choline and instead represent chance variation. In addition, the present study likely was not able to provide a strong test of the hypothesized cognitive benefits of postnatal maternal choline supplementation. It is possible that the two treatment groups did, in fact, differ in total choline breastmilk content across the intervention period, but because samples were derived from a single, full breast expression at each collection time point these differences were not captured due to natural variations in breastmilk choline concentrations. However, all women provided their samples two hours after the first feed of the day while under fasting conditions, minimizing sources of variability between women⁴⁸.

Importantly, it is unlikely that we did not detect a general benefit of postnatal maternal choline supplementation on child cognitive performance due to insensitivity of the behavioral tasks. Although there were no specific hypotheses for IQ, the SAT and the Mr. Peanut task were selected based on their ability to assess cognitive functions shown to be positively affected by maternal choline supplementation. Specifically, the SAT was selected because a study in mice showed benefits of maternal choline supplementation on a rodent analog of the task²⁷. Additionally, the Mr. Peanut task was selected because it assesses spatial

memory, a cognitive function that has consistently been shown to be improved by maternal choline supplementation¹. The sensitivity of these two tasks to variations in maternal choline intake are strongly supported by our findings of superior performance with maternal choline supplementation during pregnancy in a cohort of a similar size (**Chapters 2 & 3**).

Although the absence of group differences in total choline breastmilk concentrations in this sample precluded a strong test of the hypothesis, it would be remiss to not acknowledge the possibility that child cognition cannot be improved by maternal choline supplementation during exclusive breastfeeding. In one of the few rodent studies on this topic, postnatal choline supplementation directly to the offspring during postnatal days 1 – 15 (when pups are exclusively breastfed) had no effect on offspring cognition⁴. In contrast, postnatal choline supplementation during postnatal days 16 – 30 (when pups begin to be weaned) had beneficial effects on offspring cognition⁴. This suggests that benefits of postnatal choline supplementation may only emerge during weaning when it offsets declines in dietary choline due to the introduction of low choline foods which replace rat milk, a rich source of choline¹⁷.

The total choline content of human breastmilk may also normally meet the needs of the developing infant during exclusive breastfeeding. Although variable, the total choline metabolite concentration of human breastmilk is high^{11–16}, providing approximately two to three times more choline (per kg of body weight) than the typical adult diet⁴⁹. It is plausible that above a certain breastmilk choline concentration, no additional beneficial effects can be conferred on offspring

cognition. Furthermore, it is possible that the two, relatively high maternal choline intake levels utilized in the present study, 480 mg/d and 930 mg/d, are both sufficient to produce breastmilk choline concentrations that fully support infant brain development. It is currently unknown what minimal breastmilk choline concentration, and the maternal choline intake level needed to support it, would be adequate to prevent sub-optimal infant neurodevelopment.

Only one other published study to date has examined the relationship between breastmilk choline content and infant cognitive functioning. In this observational study (n = 55), a positive association between free choline in breastmilk at three to five months postpartum and an electrophysiological index of recognition memory was detected at six months of age in exclusively breastfed infants, but only when lutein or DHA was also high²². Because maternal diet was not assessed in this study²², it is not known whether these differences in breastmilk nutriture are a result of higher maternal choline, DHA, and lutein intake. Additionally, it is possible that the choline, DHA, and lutein content of breastmilk is associated with maternal diet during pregnancy. Therefore, the observed associations between infant memory and breastmilk nutriture could reflect, in part, maternal choline, DHA, and lutein intake during pregnancy, rather than differential postnatal intake via breastmilk. Little data exist on this subject making it unclear how likely this may be.

The present study has several strengths. First, as noted previously, the SAT and Mr. Peanut task were selected for use in this follow-up based on their ability to assess cognitive functions shown to be positively affected by maternal

choline supplementation. Therefore, our assessments should have been sensitive to the putative effects of postnatal maternal choline supplementation on child attention and spatial memory. Second, maternal diet and supplement use were highly controlled during the feeding study, providing confidence that meaningful group differences in maternal choline intake were maintained during the study period¹². Third, all testing sessions were video recorded, allowing investigators to assess child behavior during the cognitive tests and determine whether off-task behavior contributed to results.

However, the present study has two primary limitations. First, as previously discussed, total breastmilk choline concentrations at the end of the intervention period did not vary by treatment group in those successfully re-recruited. Therefore, this follow-up likely did not provide a strong test of the hypothesized benefits of maternal choline supplementation during lactation on child cognition. Second, this follow-up had a small sample size, increasing the likelihood of chance findings⁵⁰. Related to sample size, because the choline content of breastmilk is highly variable^{11,13–15} and the hypothesized benefits of postnatal maternal choline supplementation appear smaller than the benefits of prenatal choline supplementation^{3,4}, it is possible that we had inadequate statistical power to detect meaningful differences in cognitive functioning by treatment group.

In conclusion, maternal choline intake of 930 mg/d for 10 weeks during exclusive breastfeeding did not have a consistent beneficial effect on child cognitive functioning at 7 years of age relative to maternal choline intake of 480 mg/d. However, because total choline metabolite concentrations in breastmilk did not

vary by treatment group, this ancillary follow-up study does not provide a strong test of the hypothesized benefits of postnatal choline supplementation. Nonetheless, it is possible that the typically high choline content of breastmilk is already sufficient to support brain development during exclusive breastfeeding, making this period of development relatively insensitive to postnatal choline supplementation. Future RCTs should specifically target populations with a potential to benefit from postnatal choline supplementation, such as individuals with naturally low breastmilk choline levels, to provide a strong assessment of the functional effects of postnatal choline intake on child cognition.

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CHAPTER 5: CONCLUSIONS

5.1 Third Trimester Choline Supplementation & Child Cognition

This ancillary follow-up of a randomized controlled choline feeding study was conducted to address gaps in knowledge regarding the effect of prenatal choline intake on child cognitive functioning. Despite decades of research in rodents indicating the importance of maternal choline intake for offspring cognition^{1,2}, few studies in humans, observational or experimental, had been conducted on this topic and they have not provided conclusive results³⁻¹⁰. Furthermore, only two prior observational studies had assessed the relationship between prenatal choline exposure and child cognitive functioning during school-age^{5,6}. For this dissertation research we took advantage of a unique opportunity to follow-up children born to women whose diet and supplement use during the 3rd trimester were highly regulated as part of a randomized controlled choline feeding trial, the only difference between groups being total choline intake¹¹. As a result, we have gained important, preliminary data on the effects of prenatal choline supplementation on child cognition during school-age.

Summary of Findings

Data presented in **Chapter 2** provide support for the hypothesis that maternal choline supplementation during the 3rd trimester of pregnancy improves child attention at 7 years of age. Children whose mothers consumed 930 mg choline/d (approximately double the Adequate Intake [AI]) during the 3rd trimester exhibited superior attentional control on a challenging signal detection task (the

sustained attention task [SAT]) compared to children whose mothers consumed 480 mg choline/d (approximately the AI). Specifically, the pattern of results indicated children in the 930 mg/d group experienced fewer lapses in attention and had better sustained attention than children in the 480 mg/d group. Notably, this study demonstrates for the first time in humans that prenatal choline supplementation improves offspring attention based on direct behavioral assessments of the children. This finding is consistent with results from this cohort during infancy. During the first year of life, infants whose mothers were in the 930 mg/d group exhibited faster attentional orienting speed during a visual reaction time test compared to infants whose mothers were in the 480 mg/d group¹⁰, a possible developmental precursor to attentional control¹². Additionally, our results are broadly consistent with a randomized controlled trial (RCT) which detected improved cerebral inhibition in infants whose mothers were randomized to consume a choline supplement (900 mg/d) during pregnancy compared to controls⁸. This electrophysiological measure is associated with a reduced risk of attentional disorders⁸.

Data presented in **Chapter 3** provide support for the hypothesis that maternal choline supplementation during the 3rd trimester of pregnancy improves memory at 7 years of age. Children whose mothers consumed 930 mg choline/d during the 3rd trimester of pregnancy performed better on an assessment of color-location memory (the Mr. Peanut task) than children whose mothers consumed 480 mg choline/d, indicative of greater short-term spatial memory. Additionally, we administered the *Zoo locations* subtest of the Wechsler Preschool and Primary Scales of Intelligence, Fourth Edition (WPPSI-IV), an assessment of short-

term object-location memory that is less difficult than the Mr. Peanut task, due to the use of more distinct stimuli (i.e., animals). Although statistically significant group differences were not detected for *Zoo Locations*, children in the 930 mg/d group tended to exhibit better performance than children in the 480 mg/d group based on mean differences and Mr. Peanut task performance was significantly associated with *Zoo Locations* performance, providing evidence of consistency between the two measures. This finding of improved short-term spatial memory is broadly consistent with one observational study which detected a positive association between 2nd trimester choline intake and child visual memory at 7 years of age⁵. In contrast, the only other RCT to assess the effect of maternal choline supplementation on offspring memory detected no benefit during infancy for short-term visuospatial memory⁷. However, low infant compliance during testing and the collapsing of data across all levels of task difficulty suggests that it is unlikely this study provided a sensitive assessment of infant visuospatial memory.

In this study, performance on the WPPSI-IV did not vary statistically by treatment group for Full Scale IQ or other, more specific indices of child intelligence (**Chapter 3**). Because of insufficient power it would not likely have been possible to detect small but meaningful differences in IQ in this cohort. However, it was important to broadly characterize the cognitive functioning of these children. An intriguing pattern of results for the WPPSI-IV was observed such that children in the 930 mg/d group tended to exhibit better performance than the 480 mg/d group on the Working Memory Index and the Processing Speed Index, whereas differences between treatment groups were less evident for Full Scale

IQ and the Verbal Comprehension Index. Consistent with this pattern, the majority of studies that have administered tests of IQ or child development have not found associations between prenatal choline exposure and child performance on these tests⁴⁻⁶. In contrast, most observational and experimental studies that have detected a benefit of higher maternal choline intake administered tests assessing more specific cognitive functions, such as memory or processing speed^{5,8,10}.

Significance of Findings

As a result of this doctoral work, we have gained valuable, preliminary insight into the long-term effects of maternal choline supplementation during pregnancy on child cognition. Few studies have been conducted to assess the functional effects of prenatal choline exposure on offspring cognitive-behavioral functioning in humans³⁻⁹. The present study adds to the small but growing literature supporting the view that maternal choline intake during pregnancy affects child cognitive functioning. Furthermore, maternal choline intake levels needed to support fetal brain development have not been characterized. The choline AI for pregnant women was extrapolated from a single study in men pertaining to the amount of choline needed to prevent liver dysfunction¹³. The results of this doctoral work demonstrate that choline intakes of approximately double the AI produce superior child cognitive functioning compared to approximately the AI, suggesting that the current intake recommendation of 450 mg choline/d is not sufficient to support optimal child cognitive development and functioning. Although confirmation from additional, well-powered RCTs is needed, our data indicate that a re-evaluation of the choline AI for pregnant women may be warranted.

As noted previously, the present study detected a beneficial effect of higher maternal choline intake during pregnancy on child attentional control and short-term visuospatial memory. The real-world impact of prenatal choline supplementation on these functions could be significant. For example, attentional control contributes to a variety of cognitive functions including problem-solving and working memory, and is positively associated with school performance^{14–17}. Additionally, visuospatial memory has been shown to correlate with mathematical skills^{18–20} and intelligence^{21,22}.

The pattern of findings across the various cognitive domains assessed in this cohort during infancy¹⁰ and at 7 years of age suggest that prenatal choline supplementation initiates a developmental cascade that results in superior higher-order cognitive processes related to executive functioning. Executive functions are a family of cognitive processes including inhibition, working memory, and mental flexibility which facilitate problem solving and reasoning²³, and are important predictors of school readiness²⁶ and school success^{27,28}. Previous research has indicated that a developmental cascade exists such that increases in information processing speed during childhood mediate improvements in executive functions^{24,25}. Additionally, information processing abilities measured as early as infancy have been shown to predict executive functions later in childhood¹². Consistent with these linkages, information processing speed was found to be improved by prenatal choline supplementation during infancy in this cohort¹⁰ and now at 7 years of age prenatal choline supplementation was found to have beneficial effects on attentional control (**Chapter 2**), short-term visuospatial

memory (**Chapter 3**), and planning (*Nevins JEH, et al. In Preparation*).

The pattern of findings in this cohort can also be conceptualized as an effect of prenatal choline supplementation on fluid intelligence. Fluid intelligence, or an individual's ability to use abstract thought and reasoning to solve novel problems independent of acquired knowledge and skills, shares qualities with higher-order executive functions²³ and is related to cognitive functions such as attentional control and working memory^{24,29-31}. In contrast, crystallized intelligence reflects an individual's acquired knowledge and skills, which are impacted by factors such as interest, motivation, educational quality, and acculturation³². Because the mechanism by which maternal choline supplementation would affect child cognitive functioning is biological, it would be expected that tests of fluid intelligence would be more sensitive to the effects of prenatal choline supplementation than tests of crystallized intelligence, which depend largely on external, environmental factors. Although fluid and crystallized intelligence are distinct constructs, they are interrelated. Fluid intelligence influences an individual's ability to learn challenging and complex concepts and, therefore, has a functional impact on knowledge acquisition and thus crystallized intelligence³².

The potential for pregnant women and their offspring to benefit from maternal choline supplementation may be high. Average choline intake by pregnant women in North America is approximately 350 mg/d, with only approximately 10% of these women consuming choline at levels above the AI^{5,33-36}. Furthermore, among the most popular prenatal vitamins, most contain little or no choline³⁷. The two levels of choline administered in this study, 480 mg/d and 930 mg/d, are

substantially higher than the amounts most pregnant women in North America consume. Although no dose response studies have been conducted in humans, rodent research has shown that prenatal choline deprivation produces lasting cognitive dysfunction in offspring^{1,38}, in addition to the many rodent studies reporting beneficial effects of prenatal choline supplementation on offspring cognition^{1,2}. Therefore, benefits of increasing prenatal choline intake could plausibly be seen if choline intake is raised to just the AI from current average intake levels, let alone double the AI.

Future Directions

Future research into the effects of prenatal choline supplementation on child cognition should first focus on confirming the findings from this ancillary follow-up of a controlled feeding study with a large RCT designed specifically to assess child cognitive-behavioral outcomes. Although the present study provides compelling data that reveal a benefit of increased 3rd trimester choline intake on child attention and spatial memory, the sample size was small, increasing our risk of chance findings³⁹. Future RCTs should be mindful when selecting tasks to ensure they assess cognitive functions predicted by the rodent literature to be affected by maternal choline intake. More generic assessments may lead to null results because they are not sufficiently sensitive to subtle variations in the types of cognitive functions that are affected by maternal choline intake.

Beyond confirmation of the present study's findings, many questions remain regarding the effect of prenatal choline intake on child cognitive functioning. In the controlled feeding study which served as the basis for this ancillary follow-up, women were randomized to consume either 480 mg or 930 mg choline/d.

Both of these choline intake levels are higher than the average intake of pregnant women in North America (approximately 350 mg choline/d)^{5,33-36}. Therefore, the present study could not directly assess the effect of increasing prenatal choline intake from current average levels to either level administered in the study. Studies which randomize women to either a placebo or choline supplement will be needed to understand and characterize the effect of prenatal choline supplementation when maternal choline intake is moderate or low.

Another outstanding question that could not be addressed by this ancillary follow-up is the effect supplement timing. In the present study, maternal choline supplementation was limited to the 3rd trimester of pregnancy. Because important neurodevelopmental events occur throughout gestation, it is plausible that beginning maternal choline supplementation earlier in pregnancy may confer additional benefits on child cognition beyond those seen when supplementation occurs only during the 3rd trimester. Studies which assess the effect of prenatal choline supplementation beginning in the 1st or 2nd trimester are needed.

Going forward, it will be important to characterize the effects of prenatal choline supplementation on offspring cognitive-behavioral functioning across the entirety of childhood. Most studies have assessed child outcomes during infancy^{3,7,8,10} or in preschool^{4,9}. Only three studies, including the present doctoral work, have assessed the effect of prenatal choline exposure on child cognition during early school-age^{5,6}. Currently, no studies have been published on the effect of prenatal choline supplementation on offspring cognitive-behavioral functioning during adolescence. Assessments during the teenage years will allow us

to determine if benefits of prenatal choline supplementation persist beyond infancy and early childhood, as would be predicted by rodent research. Additionally, the broader impact of prenatal choline supplementation could be assessed during adolescence by examining outcomes such as scholastic success; executive functions affected by prenatal choline intake in the present study have been shown to positively correlate with school performance¹⁴⁻¹⁷ and mathematical skills¹⁸⁻²⁰.

5.2 Maternal Postnatal Choline Supplementation & Child Cognition

Although research in rodents has indicated that postnatal choline supplementation has beneficial effects on offspring cognition^{40,41}, little research on this subject has been conducted in humans. Only one prior observational study had assessed the relationship between breastmilk choline content and infant cognitive functioning⁴². No studies in humans had assessed whether maternal choline intake during breastfeeding had functional effects on child cognition. This ancillary follow-up of children whose mothers participated in a randomized controlled feeding study during the early postpartum period while exclusively breastfeeding was undertaken to address the paucity of research in this area and gain insight into the potential functional effects of maternal choline intake during lactation on child cognition.

Summary of Findings

Data presented in **Chapter 4** do not support the hypothesis that maternal choline supplementation during exclusive breastfeeding has beneficial effects on

offspring cognition at 7 years of age. Seven-year-old children whose mothers consumed 930 mg choline/d for 10 weeks while exclusively breastfeeding may have performed better on a challenging signal detection task (the SAT) than children whose mothers consumed 480 mg choline/d. However, contrary to hypotheses, children in the 480 mg/d group performed better on a test of short-term color-location memory than children in the 930 mg/d group. No differences between treatment groups were detected on any scale of the WPPSI-IV. It is unlikely that this study provided a strong test of the hypothesis that maternal choline supplementation during lactation has beneficial effects on offspring cognition, however. Specifically, among those successfully re-recruited for the 7-year follow-up, treatment groups did not vary in total breastmilk choline metabolite concentrations at the end of the intervention period. This implies that infant choline intake did not vary during the feeding study, precluding an assessment of the cognitive effects of differential postnatal choline exposure via breastmilk.

Although this study does not appear to have provided a strong test of the hypothesized benefits of higher postnatal choline intake, it must be acknowledged that there is uncertainty regarding the potential for maternal choline supplementation during lactation to affect the cognitive functioning of exclusively breastfed infants. Human breastmilk is generally high in choline^{35,43–47}, providing approximately two to three times more choline per kg of body weight to the infant than a typical adult diet⁴⁸. One study in rodents showed that postnatal choline supplementation directly to offspring during postnatal days 1-15 (when the pup is exclusively breastfeeding) had no beneficial effect on cognition compared to

controls⁴⁰. In contrast, postnatal choline supplementation to offspring during postnatal days 16-30 (when the pup is weaned) did have beneficial effects on offspring cognition⁴⁰. This implies that rat milk, also a rich source of choline, is sufficient to meet the demands of offspring neurodevelopment during this period of exclusive breastfeeding. It is possible that choline concentrations maintained in human breastmilk under a typical diet, let alone the high choline intake levels that women were randomized to in the present study, are also high enough to prevent sub-optimal infant brain development during exclusive breastfeeding.

Even if variations in breastmilk choline at the generally high levels maintained in humans are functionally important for infant cognitive development, the potential cognitive benefits of postnatal maternal choline supplementation may be difficult to detect without a substantially larger sample size than the one available for the present study. One reason for this prediction are the findings in rodents which indicate that the benefits of postnatal choline supplementation are smaller than those of prenatal choline supplementation^{40,41}. It is possible that neurodevelopmental events occurring postnatally are less sensitive to variations in choline exposure than neurodevelopmental events occurring prenatally. However, this smaller effect may also be due to the pups' continued consumption of choline-rich rat milk for substantial portions of the study period, lessening the potential benefit of postnatal choline supplementation.

Another factor relevant to our ability to detect effects of postnatal choline supplementation on offspring cognition is the variability of breastmilk choline concentrations and the extent to which variations in maternal choline intake can

affect those concentrations. Breastmilk choline concentrations are highly variable between women^{35,43–47}. Research in rodents^{49,50} and humans^{35,44} indicate that some of this variability can be explained by maternal choline intake but other factors such as genetic polymorphisms that alter choline and folate metabolism also appear to be functionally important for breastmilk choline content^{35,51}. Whether the average increase in total breastmilk choline concentrations that can be achieved by supplementing the maternal diet would be large enough to make a meaningful and detectable impact on infant neurodevelopment is unclear. Combined with the already smaller expected benefits of postnatal choline supplementation, a large sample size would likely be needed to ensure an RCT on this topic has adequate power to test the hypothesis that maternal choline supplementation during exclusive breastfeeding has beneficial effects on child cognition.

Future Directions

Our assessment of the effect of maternal choline supplementation during exclusive breastfeeding did not reveal beneficial effects on child cognition. However, this dissertation has identified several gaps in our knowledge regarding the effect of breastmilk choline content on child cognition that should be investigated in future observational and experimental studies. Notably, only two studies, including the present research, have assessed the relationship between postnatal choline intake and child cognitive functioning⁴²; very little is known about the functional effects of postnatal choline intake in humans. Observational research will be critical to characterizing the potential relationship between breastmilk choline content and cognitive functioning in exclusively breastfed infants. If breastmilk

choline concentrations do predict offspring cognitive outcomes, research may be able to identify breastmilk choline concentrations that fully support infant neurodevelopment. Conversely, possible predictors of “low” breastmilk choline content, such as maternal choline intake and genetic polymorphisms which affect choline and folate metabolism, could also be explored to help identify populations that may have the potential to benefit from postnatal choline supplementation.

Another intriguing line of research that could be followed is the role of infant choline intake when exclusive breastfeeding is discontinued and complementary feeding begins. As noted previously, one study in rodents demonstrated that postnatal choline supplementation directly to the offspring had beneficial effects on cognition during the period of weaning, but not during exclusive breastfeeding⁴⁰. Infant cognitive development may be fully supported by the levels of choline found in breastmilk, but it is also possible that the introduction of complementary foods low in choline may not fully meet the demands of neurodevelopment that continues postnatally.

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APPENDIX A: FULL TESTING PROTOCOL

A two-day testing protocol was developed to rigorously assess the effect of increased maternal choline intake on child cognition, behavior, and hypothalamic-pituitary-adrenal (HPA) axis activity at 7 years of age. The suitability of each test administered to the children was determined through a series of pilot testing sessions conducted in the 6 months prior to the start of the follow-up. Adjustments to task difficulty, instructions, and task order were made based on the experiences of study personnel administering the cognitive tests and performance of the pilot subjects to promote and enhance child motivation and performance while maintaining an appropriate level of task difficulty that would allow for differentiation between subjects.

Each child participated in approx. 3 hours of cognitive testing, split into two, 90-minute sessions held on consecutive days to ensure optimal child performance. Additionally, each testing session included two, prescheduled breaks to further prevent fatigue and maintain high levels of performance. A summary of the tasks and activities completed by the children during the testing sessions is presented in **Table A.1**.

Table A.1 Child testing protocol^a

Testing Day One	Testing Day Two
WPPSI: Zoo Locations	WPPSI: Picture Memory
Sustained Attention Task (SAT)	Attention Network Test (ANT)
Break / Saliva Collection 1	Break / Saliva Collection 1
WPPSI: Bug Search	WPPSI: Cancellation
WPPSI: Information	WPPSI: Similarities
Mr. Peanut, 1 Second RI	Mr. Peanut, 8 Second RI
Break / Height Measurement	Break / Weight Measurement
Tower of London	WPPSI: Block Design
Face Memory, Immediate Recall	WPPSI: Matrix Reasoning
Saliva Collection 2 / End of Testing	Face Memory, 24 Hour Delayed Recall
	Saliva Collection 2 / End of Testing

^aBolded tasks were analyzed as part of this dissertation

Testing sessions were conducted by two, trained graduate students blinded to the level of choline the participants' mothers received during the choline feeding study. Testers were randomized to subjects as they were enrolled in the study. To ensure adherence to the testing protocol, all cognitive testing sessions were videotaped and reviewed by study personnel. Video recordings of the sessions were also used to assess child attention and motivation during select tasks.

While children were completing the testing protocol, parents completed a series of surveys to collect demographic and health history information, and to assess child behavior, affect, and executive functioning. A summary of the surveys completed by the parents are presented in **Table A.2**.

Table A.2 Parent survey protocol

Parent Surveys Day One	Parent Surveys Day Two
Lab Saliva Collection Information	Lab Saliva Collection Information
Health History & Demographics Survey	Child Behavior Questionnaire (CBQ)
Behavior Assessment System for Children, 3 rd Edition (BASC-3)	Behavior Rating Inventory of Executive Function, 2 nd Edition (BRIEF-2)

Most testing sessions were conducted at Cornell University in the Human Metabolic Research Unit (HMRU). All children who resided in Ithaca, NY or the surrounding areas was tested at the HMRU. Additionally, the majority of children who moved from central New York and were successfully re-recruited were brought to Ithaca, NY and tested at the HMRU (pregnancy cohort: 480 mg/d, n = 2, 930 mg/d, n = 4; lactation cohort: 480 mg/d, n = 4, 930 mg/d, n = 2). Review of these sessions indicated that performance was not reduced for these children. A minority of children in the pregnancy cohort (480 mg/d: n=2; 930 mg/d: n=2) had moved from central New York and were not able to travel to Ithaca for testing. No children in the lactation cohort were tested outside of Ithaca, NY. In these cases, study personnel travelled to the participants and conducted the testing sessions in a quiet, private room near the child's residence (e.g., library conference room). Review of these sessions indicated that performance was not reduced for either the child or tester relative to testing sessions conducted at the HMRU.

APPENDIX B: SAT SUPPLEMENTARY DATA, PREGNANCY COHORT

Child sex was included as a fixed effect in all models, a decision that was made *a priori* due to research which suggests that child performance on several tests of attention varies by sex¹⁻⁵. SAT score, percent hits, and percent false alarms all varied significantly by child sex; omissions tended to vary by child sex. Specifically, females out-performed males on all measures of performance. In models which excluded child sex as a fixed effect, the direction and magnitude of differences between choline treatment groups was preserved although some comparisons lost statistical significance. For a summary of these comparisons, see **Table B.1**.

Table B.1 Comparison of primary SAT results excluding and including child sex as a fixed effect

	Child Sex Excluded			Child Sex Included		
	Estimate (95% CI)	F ^a	p	Estimate (95% CI)	F ^a	p
SAT Score						
Choline	0.13 (-0.04, 0.31)	2.60	0.12	0.15 (0.03, 0.27)	7.19	0.02
Child Sex				0.29 (0.16, 0.42)	22.19	<0.001
Percent Hits						
Choline	6.7% (-3.9%, 17.3%)	1.76	0.20	7.4% (-2.2%, 17.0%)	2.62	0.12
Choline x Block	14.6% (2.1%, 27.0%)	2.37	0.02	14.6% (2.1%, 27.0%)	2.37	0.02
Child Sex				11.2% (0.8%, 21.6%)	5.16	0.04
Percent False Alarms						
Choline	-6.6% (-23.0%, 9.8%)	0.71	0.41	-7.7% (-22.4%, 7.0%)	1.21	0.29
Choline x Block	12.7% (0.3%, 25.1%)	2.07	0.045	12.7% (0.3%, 25.1%)	2.07	0.045
Child Sex				-18.1% (-34.1%, -2.2%)	5.73	0.03
Omissions^b						
Choline	-0.44 (0.85)	0.27	0.61	-0.60 (0.82)	0.54	0.47
Child Sex				-1.59 (0.90)	3.13	0.09

^at value reported for choline by task block interactions; ^bBecause omissions were modeled using a generalized mixed model with a binomial distribution, the estimate and standard error is reported in lieu of the estimate and 95% confidence intervals

All base analyses for the SAT excluded trials due to technical difficulties (e.g., accidental minimization of task) which were extremely rare (5 out of 4320 total trials administered). Additional sensitivity analyses were conducted to determine if trials missed due to off-screen looking were important contributors to overall performance. Each table presents the results of the base analysis and the sensitivity analysis for each outcome (SAT score, **Table B.2**; percent hits, **Table B.3**; percent false alarms, **Table B.4**; omissions, **Table B.5**).

Table B.2 SAT score results from base analysis and sensitivity analysis

	Base Analysis			Sensitivity Analysis		
	Estimate (95% CI)	F ^a	p ^b	Estimate (95% CI)	F ^a	p ^b
Main Effects						
Choline	0.15 (0.03, 0.27)	7.19	0.02	0.15 (0.03, 0.27)	7.35	0.01
Signal Duration		17.17	< 0.0001		16.78	< 0.0001
17 v 29 ms	-0.14 (-0.19, -0.09)	5.18	< 0.001	-0.14 (-0.19, -0.09)	5.15	< 0.001
17 v 50 ms	-0.13 (-0.19, -0.08)	4.96	< 0.001	-0.13 (-0.19, -0.08)	4.87	< 0.001
29 v 50 ms	0.01 (-0.05, 0.06)	0.22	> 0.99	0.01 (-0.05, 0.06)	0.29	> 0.99
Block		1.52	0.23		1.40	0.26
Two-Way Interactions						
Choline x Signal Duration		0.30	0.74		0.29	0.75
Choline x Block	0.05 (-0.16, 0.26)	0.44	0.66	0.05 (-0.16, 0.26)	0.49	0.62
Signal Duration x Block		1.45	0.22		1.43	0.23
Three-Way Interaction						
Choline x Block: 17 v 29 ms	-0.02 (-0.28, 0.24)	0.16	> 0.99	-0.04 (-0.31, 0.22)	0.31	> 0.99
Choline x Block: 17 v 50 ms	-0.12 (-0.38, 0.15)	0.87	> 0.99	-0.12 (-0.39, 0.14)	0.90	> 0.99
Choline x Block: 29 v 50 ms	-0.09 (-0.36, 0.17)	0.71	> 0.99	-0.08 (-0.34, 0.19)	0.59	> 0.99

^a*t* values reported for choline x task block interaction, and all variations of the three-way interaction, due to custom contrasts used to assess the interaction of interest (differential change in performance across the session); ^bAdjusted p-values reported for pairwise comparisons and three-way interaction contrasts

Table B.3 Percent hits results from base analysis and sensitivity analysis

	Base Analysis			Sensitivity Analysis		
	Estimate (95% CI)	F ^a	p ^b	Estimate (95% CI)	F ^a	p ^b
Main Effects						
Choline	0.07 (-0.02, 0.17)	2.62	0.12	0.07 (-0.02, 0.17)	2.62	0.12
Signal Duration		29.63	< 0.0001		28.97	< 0.0001
17 v 29 ms	-0.17 (-0.21, -0.12)	6.93	< 0.001	-0.17 (-0.22, -0.12)	6.90	< 0.001
17 v 50 ms	-0.15 (-0.20, -0.11)	6.36	< 0.001	-0.15 (-0.20, -0.10)	6.24	< 0.001
29 v 50 ms	0.01 (-0.03, 0.06)	0.57	> 0.99	0.02 (-0.03, 0.06)	0.66	> 0.99
Block		4.23	0.02		4.00	0.03
One v Two	0.03 (-0.03, 0.10)	1.07	0.88	0.03 (-0.03, 0.09)	1.04	0.92
One v Three	0.09 (0.03, 0.15)	2.88	0.02	0.09 (0.02, 0.15)	2.80	0.02
Two v Three	0.06 (-0.01, 0.12)	1.81	0.24	0.05 (-0.01, 0.12)	1.76	0.26
Two-Way Interactions						
Choline x Signal Duration		0.49	0.61		0.50	0.61
Choline x Block	0.15 (0.02, 0.27)	2.37	0.02	0.15 (0.02, 0.27)	2.41	0.02
Signal Duration x Block		1.37	0.25		1.31	0.27
Three-Way Interaction						
Choline x Block: 17 v 29 ms	-0.12 (-0.35, 0.11)	1.01	0.95	-0.14 (-0.37, 0.10)	1.15	0.76
Choline x Block: 17 v 50 ms	-0.17 (-0.41, 0.06)	1.48	0.43	-0.18 (-0.41, 0.06)	1.50	0.41
Choline x Block: 29 v 50 ms	-0.06 (-0.29, 0.18)	0.47	> 0.99	-0.04 (-0.28, 0.19)	0.35	> 0.99

^a*t* values reported for choline x task block interaction, and all variations of the three-way interaction, due to custom contrasts used to assess the interaction of interest (differential change in performance across the session); ^bAdjusted p-values reported for pairwise comparisons and three-way interaction contrasts

Table B.4 Percent false alarms results from base analysis and sensitivity analysis

	Base Analysis			Sensitivity Analysis		
	Estimate (95% CI)	F ^a	p	Estimate (95% CI)	F ^a	p
Main Effects						
Choline	-0.08 (-0.22, 0.07)	1.21	0.29	-0.08 (-0.22, 0.07)	1.22	0.28
Block		0.40	0.67		0.37	0.70
Two-Way Interaction						
Choline x Block	0.13 (0.003, 0.25)	2.07	0.045	0.13 (0.003, 0.25)	2.08	0.045

^at values reported for choline x task block interaction due to custom contrasts used to assess the interaction of interest (differential change in performance across the session);

^bAdjusted p-values reported for pairwise comparisons

Table B.5 Omissions results from base analysis and sensitivity analysis

	Base Analysis			Sensitivity Analysis		
	Estimate (SEM)	F ^a	p ^b	Estimate (SEM)	F ^a	p ^b
Main Effects						
Choline	-0.60 (0.82)	0.54	0.47	-0.65 (0.83)	0.62	0.44
Signal Duration		53.01	< 0.0001		53.72	< 0.0001
Block		0.25	0.78		0.34	0.71
Two-Way Interactions						
Choline x Signal Duration		0.91	0.44		0.60	0.62
Choline x Block	0.67 (0.58)	1.14	0.26	0.77 (0.59)	1.30	0.20
Signal Duration x Block		0.98	0.44		1.09	0.37
Three-Way Interaction						
Choline x Block: Non v 17 ms	0.01 (0.84)	0.01	> 0.99	0.30 (0.86)	0.35	> 0.99
Choline x Block: Non v 29 ms	0.30 (1.2)	0.25	> 0.99	0.27 (1.2)	0.22	> 0.99
Choline x Block: Non v 50 ms	-0.21 (1.55)	0.14	> 0.99	-0.24 (1.55)	0.16	> 0.99
Choline x Block: 17 v 29 ms	0.29 (1.38)	0.21	> 0.99	-0.03 (1.39)	0.02	> 0.99
Choline x Block: 17 v 50 ms	-0.23 (1.69)	0.13	> 0.99	-0.54 (1.70)	0.32	> 0.99
Choline x Block: 29 v 50 ms	-0.51 (1.89)	0.27	> 0.99	-0.51 (1.90)	0.27	> 0.99

^a*t* values reported for choline x task block interaction, and all variations of the three-way interaction, due to custom contrasts used to assess the interaction of interest (differential change in performance across the session); ^bAdjusted p-values reported for three-way interaction contrasts

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APPENDIX C: MR. PEANUT TASK MODIFICATIONS

The Mr. Peanut task was specifically designed for use in children^{1,2}. However, pilot testing conducted by our group in 7-year-old children suggested that the task as programmed in Inquisit 5 (Millisecond Software, Seattle, WA) would be too difficult, producing data with low variability and potentially precluding the detection of group differences if they existed. To address this concern, several modifications were made to the task.

First, the number of possible colors for the dots was reduced from seven to five. The two colors removed (gold and maroon) were similar to others included in the task (yellow and red). Therefore, their exclusion helped to increase discriminability between the five remaining colors. Second, the number of potential locations for dots was reduced from 14 to 10. The original Mr. Peanut cartoon included spaces for dots on the antennae and cheeks which were removed for use in the follow-up study (see **Figure C**). By removing these locations, the total area needed to scan was reduced and the space between locations was increased, the overall effect being decreased difficulty. Third, the number of trials per level was increased from three to four. The effect of this change was two-fold: 1) it increased the number of opportunities a child had to pass an individual level and 2) it increased the maximum number of trials administered to 20, an amount similar to the maximum in the original task (21 trials).

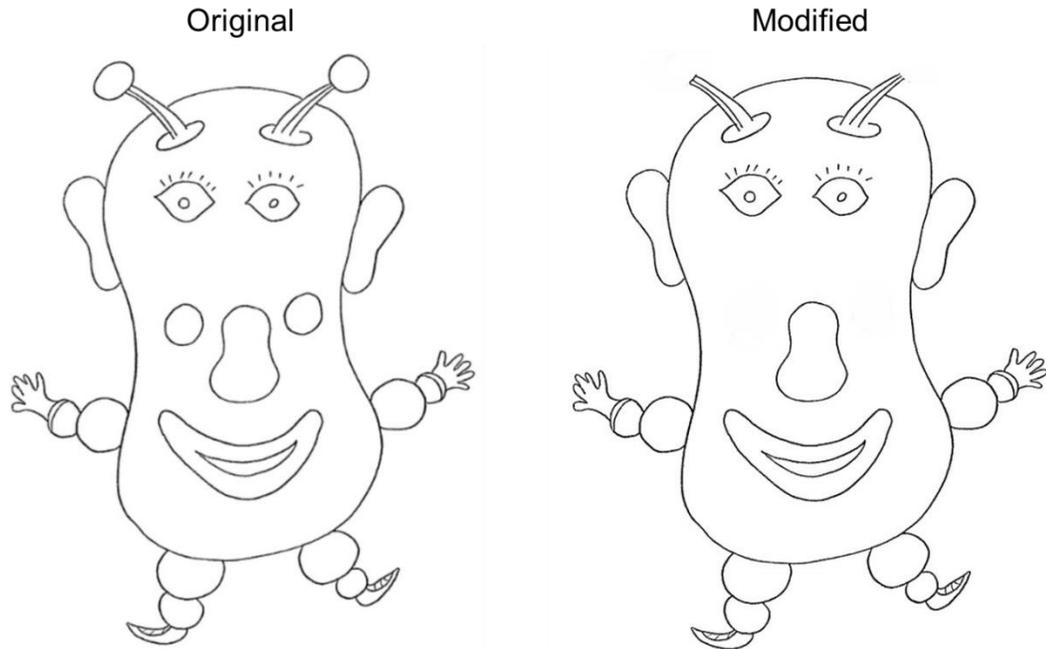


Figure C Comparison between original and modified Mr. Peanut cartoon

In addition to modifications made to the Mr. Peanut task to reduce difficulty, a second version of task was programmed with the purpose of tapping memory functions expected to be dependent on the hippocampus, an area of the brain consistently shown to be modified by maternal choline supplementation^{3,4}. In the original task, the retention interval between the presentation and recall phases was 1 s, assessing spatial working memory. The second version was programmed to include an 8 s retention interval between the presentation and recall phases. To prevent rehearsal during the 8 s retention interval, the children were instructed to count aloud with study personnel.

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APPENDIX D: MR. PEANUT SUPPLEMENTARY DATA, PREGNANCY CO- HORT

One participant in the 480 mg/d group missed a full presentation phase for one trial (one dot, 8 s retention interval) due to an individual entering the testing room. The only outcome affected by this interruption was total trials correct as the child had already passed a trial for the first level, ensuring they would move on to the second level. The analysis of total trials correct was run using data as collected (in which the child received no credit for the trial) and data which gave the child full credit for the trial. Comparisons of these models are presented in **Table D.1**. Because the child had gotten all other one dot trials correct (for both the 1 and 8 s retention interval conditions) and the models produced virtually identical results, the analyses which awarded the child full credit for the missed trial were presented in the main paper.

Table D.1 Comparison of results for total trials correct from analyses which did and did not give full credit for missed trial

	No Credit			Full Credit		
	Estimate (95% CI)	F ^a	p ^b	Estimate (95% CI)	F ^a	p ^b
Choline	1.5 (-0.4, 3.4)	2.82	0.11	1.5 (-0.5, 3.4)	2.52	0.13
RI	3.6 (2.4, 4.9)	37.97	< 0.0001	3.6 (2.3, 4.8)	34.40	< 0.0001
Choline x RI		1.57	0.23		1.70	0.21
<i>At 1 s</i>	2.3 (0.1, 4.5)	2.09	0.09	2.3 (-0.003, 4.5)	2.04 ¹	0.10
<i>At 8 s</i>	0.8 (-1.4, 3.0)	0.73	0.94	0.7 (-1.6, 2.9)	0.61 ¹	> 0.99

^a*t* value reported for contrasts at each RI for the treatment group by RI interaction. ^bAdjusted p-value reported for contrasts at each RI for the treatment group by RI interaction.

Child sex was included as a fixed effect in all models, a decision that was made *a priori* due to research which suggests that spatial memory varies by sex in children and adults¹⁻⁴. Highest level passed tended to vary by child sex ($p = 0.14$); a similar pattern was present for the number of trials correct. Specifically, females tended to perform better than males on both measures. In models which excluded child sex as a fixed effect, the direction and magnitude of differences between choline treatment groups was preserved although some comparisons lost statistical significance (i.e., main effect of choline on highest level passed). For a summary of these comparisons, see **Table D.2**.

Table D.2 Results with child sex excluded and included as a fixed effect

	Child Sex Excluded			Child Sex Included		
	Estimate (95% CI)	F ^a	p ^b	Estimate (95% CI)	F ^a	p ^b
Number of Trials Correct						
Choline	1.4 (-0.5, 3.3)	2.46	0.13	1.5 (-0.5, 3.4)	2.52	0.13
Choline x RI		1.70	0.21		1.70	0.21
At 1 s	2.2 (-0.1, 4.5)	2.03	0.10	2.3 (-0.003, 4.5)	2.04	0.10
At 8 s	0.6 (-1.6, 2.9)	0.58	>0.99	0.7 (-1.6, 2.9)	0.61	>0.99
Child Sex				0.7 (-1.4, 2.8)	0.47	0.50
Highest Level						
Choline	0.64 (-0.05, 1.35)	3.82	0.07	0.67 (0.01, 1.34)	4.53	0.048
Choline x RI		1.11	0.31		1.11	0.31
At 1 s	0.89 (0.07, 1.71)	2.20	0.06	0.92 (0.12, 1.73)	2.34	0.052
At 8 s	0.39 (-0.43, 1.22)	0.98	0.67	0.43 (-0.38, 1.23)	1.08	0.58
Child Sex				0.54 (-0.19, 1.26)	2.43	0.14

^a*t* values reported for contrasts at each RI for the treatment group by RI interaction. ^bAdjusted *p*-values reported for contrasts at each RI for the treatment group by RI interaction.

Works Cited, Appendix D

- 1 Leon I, Cimadevilla JM, Tascon L. Developmental gender differences in children in a virtual spatial memory task. *Neuropsychology* 2014; **28**: 485–95.
- 2 Postma A, Jager G, Kessels RPC, Koppeschaar HPF, Van Honk J. Sex differences for selective forms of spatial memory. *Brain Cogn* 2004; **54**: 24–34.

- 3 Newhouse P, Newhouse C, Astur RS. Sex differences in visual-spatial learning using a virtual water maze in pre-pubertal children. *Behav Brain Res* 2007; **183**: 1–7.
- 4 Postma A, Izendoorn R, De Haan EHF. Sex Differences in Object Location Memory. *Brain Cogn* 1998; **36**: 334–45.

APPENDIX E: SAT SUPPLEMENTARY DATA, LACTATION COHORT

Child sex was included as a fixed effect in all models, a decision that was made *a priori* due to research which suggests that child performance on several tests of attention varies by sex¹⁻⁵. In no model was a statistically significant effect of child sex detected. However, females generally exhibited better performance than males as assessed by SAT score, percent hits, and percent false alarms, consistent with the pregnancy cohort. Regarding the effect of maternal choline on SAT performance, the inclusion of child sex did not alter conclusions (see **Figure E.1**)

Figure E.1 Comparison of primary SAT results excluding or including child sex as a fixed effect

	Child Sex Excluded			Child Sex Included		
	Estimate (95% CI)	F ^a	p	Estimate (95% CI)	F ^a	p
SAT Score						
Choline	0.10 (-0.12, 0.31)	0.92	0.35	0.07 (-0.15, 0.29)	0.46	0.51
Choline x Signal Duration		3.24	0.04		3.24	0.04
Child Sex				-0.10 (-0.33, 0.12)	0.97	0.34
Percent Hits						
Choline	0.05 (-0.05, 0.15)	1.14	0.30	0.03 (-0.07, 0.14)	0.49	0.49
Choline x Signal Duration		3.00	0.054		3.00	0.054
Child Sex				-0.07 (-0.17, 0.03)	2.06	0.17
Percent False Alarms						
Choline	-0.04 (-0.16, 0.08)	0.48	0.50	-0.03 (-0.16, 0.10)	0.26	0.62
Child Sex				0.04 (-0.10, 0.17)	0.32	0.58
Omissions^b						
Choline	0.05 (0.47)	0.01	0.91	-0.04 (0.49)	0.01	0.93
Child Sex				-0.46 (0.48)	0.91	0.36

^at value reported for choline by task block interactions; ^bBecause omissions were modeled using a generalized mixed model with a binomial distribution, the estimate and standard error is reported in lieu of the estimate and 95% confidence intervals

Additional sensitivity analyses were conducted to determine if trials missed due to off-screen looking were important contributors to overall performance. Each table presents the results of the base analysis and the sensitivity analysis for each outcome (SAT score, **Table E.2**; percent hits, **Table E.3**; percent false alarms, **Table E.4**; omissions, **Table E.5**). In no case were conclusions altered by this analysis.

Table E.2 SAT score results from base analysis and sensitivity analysis

	Base Analysis			Sensitivity Analysis		
	Estimate (95% CI)	F ^a	p ^b	Estimate (95% CI)	F ^a	p ^b
Main Effects						
Choline	0.07 (-0.15, 0.29)	0.46	0.51	0.08 (-0.14, 0.29)	0.54	0.47
Signal Duration		26.68	< 0.0001		27.42	< 0.0001
Block		2.96	0.07		2.98	0.07
Two-Way Interactions						
Choline x Signal Duration		3.24	0.04		2.86	0.06
<i>At 17 ms</i>	0.12 (-0.11, 0.34)	1.12	0.84	0.12 (-0.10, 0.34)	1.16	0.79
<i>At 29 ms</i>	0.02 (-0.21, 0.24)	0.16	> 0.99	0.03 (-0.19, 0.25)	0.26	> 0.99
<i>At 50 ms</i>	0.07 (-0.15, 0.30)	0.69	> 0.99	0.08 (-0.14, 0.30)	0.75	> 0.99
Choline x Block	0.03 (-0.17, 0.22)	0.30	0.77	0.03 (-0.16, 0.23)	0.35	0.73
Signal Duration x Block		0.48	0.75		0.43	0.79
Three-Way Interaction						
Choline x Block: 17 v 29 ms	-0.07 (-0.27, 0.12)	0.75	> 0.99	-0.11 (-0.30, 0.08)	1.11	0.81
Choline x Block: 17 v 50 ms	0.09 (-0.10, 0.29)	0.96	> 0.99	0.10 (-0.9, 0.29)	1.01	0.95
Choline x Block: 29 v 50 ms	0.17 (-0.03, 0.36)	1.71	0.27	0.20 (0.01, 0.40)	2.11	0.11

^a*t* values reported for choline x task block interaction, and all variations of the three-way interaction, due to custom contrasts used to assess the interaction of interest (differential change in performance across the session). ^bAdjusted p-values reported for pairwise comparisons and three-way interaction contrasts

Table E.3 Percent hits results from base analysis and sensitivity analysis

	Base Analysis			Sensitivity Analysis		
	Estimate (95% CI)	F ^a	p ^b	Estimate (95% CI)	F ^a	p ^b
Main Effects						
Choline	0.03 (-0.07, 0.14)	0.49	0.49		0.62	0.44
Signal Duration		29.73	< 0.0001		30.48	< 0.0001
Block		2.95	0.07		2.83	0.07
Two-Way Interactions						
Choline x Signal Duration		3.00	0.05		2.66	0.08
<i>At 17 ms</i>	0.08 (-0.03, 0.19)	1.54	0.41	0.08 (-0.3, 0.19)	1.57	0.39
<i>At 29 ms</i>	-0.02 (-0.13, 0.09)	0.36	> 0.99	-0.01 (-0.12, 0.10)	0.21	> 0.99
<i>At 50 ms</i>	0.04 (-0.07, 0.15)	0.69	> 0.99	0.04 (-0.07, 0.15)	0.76	> 0.99
Choline x Block	0.04 (-0.08, 0.16)	0.65	0.52	0.04 (-0.09, 0.17)	0.62	0.54
Signal Duration x Block		0.44	0.78		0.41	0.80
Three-Way Interaction						
Choline x Block: 17 v 29 ms	-0.06 (-0.27, 0.14)	0.63	> 0.99	-0.09 (-0.29, 0.11)	0.92	> 0.99
Choline x Block: 17 v 50 ms	0.11 (-0.09, 0.31)	1.10	0.83	0.11 (-0.09, 0.31)	1.12	0.79
Choline x Block: 29 v 50 ms	0.18 (-0.03, 0.38)	1.73	0.26	0.21 (0.01, 0.41)	2.04	0.13

^a*t* values reported for choline x task block interaction, and all variations of the three-way interaction, due to custom contrasts used to assess the interaction of interest (differential change in performance across the session). ^bAdjusted p-values reported for pairwise comparisons and three-way interaction contrasts

Table E.4 Percent false alarms results from base analysis and sensitivity analysis

	Base Analysis			Sensitivity Analysis		
	Estimate (95% CI)	F ^a	p	Estimate (95% CI)	F ^a	p
Main Effects						
Choline	-0.03 (-0.16, 0.10)	0.26	0.62	-0.03 (-0.16, 0.10)	0.28	0.61
Block		2.00	0.15		2.32	0.11
Two-Way Interaction						
Choline x Block	0.02 (-0.07, 0.11)	0.41	0.68	0.01 (-0.08, 0.10)	0.32	0.75

^at values reported for choline x task block interaction due to custom contrast used to assess the interaction of interest (differential change in performance across the session).

Table E.4 Omissions results from base analysis and sensitivity analysis

	Base Analysis			Sensitivity Analysis		
	Estimate (SEM)	F ^a	p	Estimate (SEM)	F ^a	p
Main Effects						
Choline	-0.04 (0.49)	0.01	0.93	-0.01 (0.52)	0.00	0.98
Signal Duration		10.66	< 0.0001		10.88	< 0.0001
Block		1.00	0.37		0.63	0.54
Two-Way Interactions						
Choline x Signal Duration		0.20	0.90		0.28	0.84
Choline x Block	-0.14 (0.59)	0.23	0.82	-0.10 (0.61)	0.16	0.87
Signal Duration x Block		1.72	0.12		1.88	0.09

^at values reported for choline x task block interaction due to custom contrast used to assess the interaction of interest (differential change in performance across the session).

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- 1 Lin CCH, Hsiao CK, Chen WJ. Development of sustained attention assessed using the Continuous Performance Test among children 6-15 years of age. *J Abnorm Child Psychol* 1999; **27**: 403–12.
- 2 Gur RC, Richard J, Calkins ME, *et al.* Age group and sex differences in performance on a computerized neurocognitive battery in children age 8-21. *Neuropsychology* 2012; **26**: 251–65.
- 3 Groot AS, de Sonnevile LMJ, Stins JF, Boomsma DI. Familial influences on sustained attention and inhibition in preschoolers. *J Child Psychol Psychiatry* 2004; **45**: 306–14.

- 4 Klenberg L, Korkman M, Lahti-Nuutila P. Differential development of attention and executive functions in 3- to 12-year-old Finnish children. *Dev Neuropsychol* 2001; **20**: 407–28.
- 5 Greenberg LM, Waldmant ID. Developmental Normative Data on The Test of Variables of Attention (T.O.V.A.TM). *J Child Psychol Psychiatry* 1993; **34**: 1019–30.

APPENDIX F: MR. PEANUT SUPPLEMENTARY DATA, LACTATION CO- HORT

Child sex was included as a fixed effect in all models, a decision that was made *a priori* due to research which suggests that spatial memory varies by sex in children and adults¹⁻⁴. Performance did not vary significantly by child sex for highest level passed or the number of trials correct. In models which excluded child sex as a fixed effect, the direction and magnitude of differences between choline treatment groups were preserved. For a summary of these comparisons, see **Table F**.

Table F Results with child sex excluded and included as a fixed effect

	Child Sex Excluded			Child Sex Included		
	Estimate (95% CI)	F ^a	p ^b	Estimate (95% CI)	F ^a	p ^b
Number of Trials Correct						
Choline	-0.78 (-2.48, 0.93)	0.93	0.35	-0.88 (-2.70, 0.93)	1.08	0.31
Choline x RI		4.68	0.046		4.68	0.046
At 1 s	-0.23 (-1.99, 1.54)	0.27	> 0.99	-0.33 (-2.20, 1.53)	0.38	> 0.99
At 8 s	-1.33 (-3.09, 0.44)	1.57	0.26	-1.43 (-3.30, 0.43)	1.62	0.24
Child Sex				-0.44 (-2.28, 1.41)	0.26	0.62
Highest Level Passed						
Choline	-0.46 (-0.93, 0.01)	4.35	0.05	-0.50 (-1.00, -0.004)	4.62	0.048
Choline x RI		0.99	0.33		0.99	0.33
At 1 s	-0.25 (-0.88, 0.38)	0.81	0.84	-0.29 (-0.93, 0.36)	0.91	0.74
At 8 s	-0.68 (-1.30, -0.05)	2.19	0.07	-0.71 (-1.36, -0.07)	2.26	0.06
Child Sex				-0.16 (-0.66, 0.35)	0.43	0.52

^at values reported for contrasts at each RI for the treatment group by RI interaction.

^bAdjusted p-values reported for contrasts at each RI for the treatment group by RI interaction.

Works Cited, Appendix F

- 1 Leon I, Cimadevilla JM, Tascon L. Developmental gender differences in children in a virtual spatial memory task. *Neuropsychology* 2014; **28**: 485–95.
- 2 Postma A, Jager G, Kessels RPC, Koppeschaar HPF, Van Honk J. Sex

differences for selective forms of spatial memory. *Brain Cogn* 2004; **54**: 24–34.

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- 4 Postma A, Izendoorn R, De Haan EHF. Sex Differences in Object Location Memory. *Brain Cogn* 1998; **36**: 334–45.

APPENDIX G: POST-HOC OBSERVED POWER CALCULATIONS

Observed power was calculated for the primary outcome of each task. Because mixed models were used to assess performance on the sustained attention task (SAT) and the Mr. Peanut color-location memory task, observed power was estimated using the R (Version 3.5.1) package SIMR¹. SIMR estimates power for generalized linear mixed models using Monte Carlo simulations¹. Child performance on the WPPSI-IV was assessed with a general linear model, therefore observed power was calculated using PROC GLMPOWER in SAS (Version 9.4, Cary, NC).

Pregnancy Cohort

Models for the primary outcomes of interest for the SAT and Mr. Peanut contained interaction terms with task parameters (e.g., task block, signal duration). Because SIMR cannot estimate power for main effects when interaction terms are present in the model and because the main effect of treatment group was, in general, the comparison of interest, models were simplified to only include main effects. This approach was deemed appropriate because no significant interactions were detected for either SAT score or highest level passed, and the removal of interaction terms did not change the effect estimates. In all cases, 1100 simulations were run (seed number 4718) to ensure at least 1000 simulations with no warnings or errors were obtained.

Observed power for SAT score (task: SAT) was estimated to be 71.91% (95% CI: [69.15%, 74.55%]). The 1100 simulations yielded 75 warnings (model

failed to converge). When simulations with warnings were dropped, observed power was estimated to be 71.61%.

Observed power for highest level passed (task: Mr. Peanut color-location memory) was estimated to be 52.36% (95% CI: [49.36%, 55.35%]). The 1100 simulations yielded 4 warnings (model failed to converge). When simulations with warnings were dropped, observed power was estimated to be 52.28%.

Observed power for Full Scale IQ (task: WPPSI-IV) was calculated to be 14.9%. Based on our observed results, a total sample size of 171 individuals would be required to detect statistically significant differences between treatment groups with 80% power.

Lactation Cohort

Models for the primary outcomes of interest for the SAT and Mr. Peanut contained interaction terms with task parameters (e.g., task block, signal duration). This approach was deemed appropriate for highest level passed because no significant interactions were detected, and the removal of the interaction term did not change the effect estimate. Although a statistically significant interaction between treatment group and signal duration was present, pairwise comparisons indicated no statistically significant differences between treatment groups were present; therefore, the same approach of removing interaction terms was followed. In all cases, 1100 simulations were run (seed number 4718) to ensure at least 1000 simulations with no warnings or errors were obtained.

Observed power for SAT score (task: SAT) was estimated to be 8.91% (95% CI: [7.29%, 10.75%]). The 1100 simulations yielded 70 warnings (model

failed to converge). When simulations with warnings were dropped, observed power was estimated to be 9.03%.

Observed power for highest level passed (task: Mr. Peanut color-location memory) was estimated to be 49.45% (95% CI: [46.46%, 52.45%]). The 1100 simulations yielded 4 warnings (model failed to converge). When simulations with warnings were dropped, observed power was estimated to be 49.54%.

Observed power for Full Scale IQ (task: WPPSI-IV) was calculated to be 6.1%. Based on our observed results, a total sample size of 1314 individuals would be required to detect statistically significant differences between treatment groups with 80% power.

Works Cited, Appendix G

- 1 Green P, MacLeod CJ. SIMR : an R package for power analysis of generalized linear mixed models by simulation. *Methods Ecol Evol* 2016; **7**: 493–8.