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Prenatal choline, cannabis, and infection, and their association with offspring development of attention and social problems through 4 years of age

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Abstract

Background. Prenatal choline is a key nutrient, like folic acid and vitamin D, for fetal brain development and subsequent mental function. We sought to determine whether effects of higher maternal plasma choline concentrations on childhood attention and social problems, found in an initial clinical trial of choline supplementation, are observed in a second cohort.
Methods. Of 183 mothers enrolled from an urban safety net hospital clinic, 162 complied with gestational assessments and brought their newborns for study at 1 month of age; 83 continued assessments through 4 years of age. Effects of maternal 16 weeks of gestation plasma choline concentrations ⩾7.07 μM, 1 S.D. below the mean level obtained with supplementation in the previous trial, were compared to lower levels. The Attention Problems and Withdrawn Syndrome scales on Child Behavior Checklist 1½–5 were the principal outcomes.
Results. Higher maternal plasma choline was associated with lower mean Attention Problems percentiles in children, and for male children, with lower Withdrawn percentiles. Higher plasma choline concentrations also reduced Attention Problems percentiles for children of mothers who used cannabis during gestation as well as children of mothers who had gestational infection.
Conclusions. Prenatal choline's positive associations with early childhood behaviors are found in a second, more diverse cohort. Increases in attention problems and social withdrawal in early childhood are associated with later mental illnesses including attention deficit disorder and schizophrenia. Choline concentrations in the pregnant women in this study replicate other research findings suggesting that most pregnant women do not have adequate choline in their diets.

Prenatal origins of several mental illnesses have been established from epidemiological studies of famines and pandemics, and observational studies of behavioral deficit newborns who later develop mental illness (Brown & Derkits, 2010; Erlenmeyer-Kimling & Cornblatt, 1987; Mednick, Machon, Huttunen, & Bonett, 1988; Susser & Lin, 1992; Walker, Savoie, & Davis, 1994). Many genes associated with both brain development and mental illnesses are more highly expressed before birth than afterward (Birnbaum, Jaffe, Hyde, Kleinman, & Weinberger, 2014). The implication is that fetal brain development is a critical period of risk for later mental illness. Prenatal folic acid supplementation is a paradigmatic example of a treatment to prevent fetal abnormalities that are difficult to treat postnatally (Wald, Sneddon, Densem, Frost, & Stone, 1992). Vitamin D and choline are being similarly considered as possible prenatal interventions for mental illness (McGrath et al., 2010; Zeisel, 2006a).

Choline is concentrated in the amniotic fluid from the maternal plasma by active transport in the placenta (Zeisel, Epstein, & Wurtman, 1980). Some choline is synthesized by the mother, but the majority comes from dietary sources (Zeisel & da Costa, 2009). In addition to its roles as a major methyl donor along with B_{12}, betaine, and folate in DNA methylation and as the synthetic precursor to the phospholipids necessary for membrane stabilization, it is involved in neurotransmission (Albright, Tsai, Friedrich, Mar, & Zeisel, 1999; Ballard, Sun, & Ko, 2012; Gossell-Williams, Fletcher, McFarlane-Anderson, Jacob, & Zeisel, 2005; Zeisel, 2000; Zeisel & da Costa, 2009), both as a direct agonist at cholinergic receptors and as the precursor to acetylcholine (Zeisel, 2006b; Zeisel et al., 1980). Choline's agonist role requires the highest amniotic concentrations (Frazier et al., 1998). One-carbon metabolism and membrane

synthesis in the fetus can proceed at lower concentrations, and if the available choline supply is limited, these pathways consume most of the available choline. Therefore, maternal plasma choline levels are hypothesized to be a critical factor in the activation of cholinergic receptors during fetal brain development.

Cerebral interneurons are migrating into the cerebral plate and differentiating during the 16th week of gestation, when maternal choline levels were assessed in this study (Vasistha et al., 2019). Optogenetic inactivation of these interneurons in animal models is associated with decreased attention and social behavior (Yizhar et al., 2011). Activation of $\alpha 7$-nicotinic acetylcholine receptors by choline in the amniotic fluid facilitates the transition of the embryonic KCCN chloride pump to the mature KCC2 chloride pump, which increases the chloride gradient across the neuronal cell membrane and allows GABA to become an inhibitory neurotransmitter (Liu, Neff, & Berg, 2006). Expression of $\alpha 7$-nicotinic receptors is higher before birth than in newborns or adults, consistent with their major role in the maturation of GABAergic transmission (Court et al., 1997). However, cholinergic innervation has not yet reached the cerebrum from the midbrain (Descarries, Aznavour, & Hamel, 2005). Choline in the amniotic fluid is needed in early development to activate $\alpha 7$-nicotinic acetylcholine receptors to ensure maturation of chloride pumps and GABAergic neurotransmission (Frazier et al., 1998). This maturation process is not fully complete in patients with schizophrenia (Hyde et al., 2011).

We conducted a double-blind, randomized trial of prenatal choline supplementation beginning by 15 weeks of gestation that compared 7300 mg phosphatidylcholine, equivalent to 900 mg choline, to placebo to test the hypothesis that choline supplementation would enhance the development of cerebral inhibitory function (Ross et al., 2013). Supplementation continued after birth with blinded treatment of the newborn until 52 gestational weeks of age. The development of cerebral inhibition was assessed in 1-month-old newborns by the inhibition of the P50-evoked potential to a repeated auditory stimulus. P50 auditory-evoked potentials have a source in the hippocampus (Goff, Williamson, VanGilder, Allison, & Fisher, 1980). In animal models of the P50-evoked potential, the activity of hippocampal GABAergic interneurons is responsible for the inhibition to repeated stimuli (Miller & Freedman, 1995). Choline supplementation significantly enhanced the development of P50 inhibition, as assessed in the newborns. A subsequent report demonstrated that the decreased development of P50 inhibition was related to later childhood behavior problems on the Child Behavior Checklist 1½–5 (CBCL1½–5) (Ross et al., 2016). Mean rating percentiles on the Attention Problems scale were significantly lower for those who received supplement (59.4, S.E. = 2.5) compared to those who received placebo (65.9, S.E. = 2.3); there was no effect of gender. Ratings on the Withdrawn Syndrome scale showed effects of supplement and gender. Overall, those who received supplement had lower mean rating percentiles than those who received placebo, and mean rating percentiles for females were lower compared to males. For males who received supplement, mean rating percentiles were significantly lower (60.2, S.E. = 3.2) compared to ratings for those who received placebo (69.4, S.E. = 3.4); this difference was not significant for females.

Other studies of the effects of prenatal choline on outcomes in early childhood include three double-blind, placebo-controlled trials and two observational studies. One trial reported decreased reactive saccadic latency from 4 to 13 months of age in children of healthy mothers who received third trimester choline supplementation, a finding which the authors interpreted as increased processing speed (Caudill, Strupp, Muscalu, Nevins, & Canfield, 2018). Another trial of mid-pregnancy choline supplementation in women who had heavy alcohol use found increased novelty preference scores at 12 months of age on the Fagan Test of Infant Intelligence, which the authors interpreted as better visual recognition memory (Jacobson et al., 2018). A trial of phosphatidylcholine supplementation in healthy women beginning at 18 weeks of gestation found no effect on the Visuospatial Memory Delayed Response Task, the Mac-Arthur-Bates Short Form Vocabulary Checklist: Level I, or the Mullen Scales of Early Learning at 10–12 months of age placebo; scores were above the normal range for offspring of both placebo and supplemented mothers (Cheatham et al., 2012). Higher maternal choline concentrations in non-randomized observational studies were associated with increased cognition scores at 18 months of age on the Bayley Scales of Infant Development (Wu, Dyer, King, Richardson, & Innis, 2012). The longest duration study, based on the assessment of choline in the maternal diet in the first and second trimesters, found increased performance on the Wide Range Assessment of Memory and Learning, Design and Picture Memory subtests, in the 7-year-old offspring (Boeke et al., 2013).

The current study was undertaken to expand the results of the initial trial of choline supplementation: (1) mothers who had infections or used substances including nicotine, alcohol, and cannabis, were included, whereas they were excluded from the initial trial, (2) to obtain a more diverse population, the women were not randomized or required to take supplements, because the increased compliance requirements exclude many women. All women received dietary instruction as in the previous trial. Maternal plasma choline concentrations obtained with the phosphatidylcholine supplement in the randomized trial were 15.21 μM (S.D. 8.14), compared to 7.85 μM (S.D. 2.54) for placebo ($p = 0.03$). Choline concentrations $\geqslant$ 7.07 μM, 1 S.D. below the mean level obtained with phosphatidylcholine supplements in the previous trial, were used in the current analysis as a comparator to the supplement. The children have now reached 4 years of age. This paper analyzes their CBCL½–5 Attention Problems and Withdrawn Syndrome scales to assess the hypothesis that higher choline levels in this second cohort would be associated with decreased scores on the Attention Problems and Withdrawn Syndrome subscales, particularly in male offspring, as found in the initial trial of choline supplementation (Ross et al., 2016). We also hypothesized, based on the previous trial, that the effects on CBCL½–5 problems would be related to the children's development of P50 inhibition, assessed when they were newborns.

Previous publications from this second cohort have shown interaction of higher choline concentrations with the adverse effects of infection and cannabis on the child through 52 weeks of gestational age (Freedman et al., 2019; Hoffman et al., 2020). In these studies, higher maternal choline concentrations were associated with improved newborn P50 cerebral auditory-evoked response inhibition as well as improved outcomes on the Orienting/Regulation Index of the Infant Behavior Questionnaire-R (IBQ-R) (Gartstein & Rothbart, 2003) at 52 weeks of gestational age. Reduced scores on the Orienting/Regulation Index of the IBQ-R are related to poorer performance on measures of reading readiness at 4 years of age and increased distractibility at 9 years of age (Gartstein, Putnam, & Kliewer, 2016). The pathogenic mechanism for most infections appears to be the effects of the mother's inflammatory response on the

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placenta and fetus. In animal models of maternal immune activation effects, dietary choline supplementation counteracts the increase in interleukin-6 in the fetal brain and the increased anxiety behaviors in the offspring (Wu et al., 2015). Cannabis effects on the developing brain may be related to the co-location of CB1 receptors on the same interneurons as α7-nicotinic receptors (Morales, Hein, & Vogel, 2008). Blockade of CB1 receptors by cannabis interferes with endogenous cannabinoid signaling that promotes neurite outgrowth. The effects of α7-nicotinic receptor activation on GABAergic function appear to offset the adverse effects of cannabis. We hypothesized that the interactions of maternal choline levels with either infection or cannabis use would also be associated with decreased CBCL½–5 Attention Problems through 4 years of age.

Methods

Subjects

Women were enrolled from a public safety-net prenatal clinic at 14–16 weeks of gestation from July 2013 until July 2016. Gestational age was established by first ultrasound (ACOG, 2017). Exclusions were fetal anomaly and major maternal medical morbidity. The Colorado Multiple Institution Review Board approved the study; all mothers, and fathers if available, gave informed consent. Of 183 mothers enrolled, 162 complied with gestational assessments and brought their newborns for study at 1 month of age. For assessments through 4 years of age, 83 women continued participation at various time points. The proportion of women with higher gestational choline concentrations did not change significantly between prenatal and later assessments. Assessment details are provided in online Supplement S1.

Gestational choline measurement

Choline and other metabolite plasma levels were measured 2–3 h after a meal at 16 and 28 weeks of gestation. Choline was the principal measure because it is required for fetal nicotinic receptor activation, which is involved in interneuron development (Ross et al., 2016). Dietary content was not assessed because of the limited relationship of assessments to serum levels (Abratte et al., 2009; Wu et al., 2012). Maternal levels obtained in non-fasting conditions, as in the current study, are elevated only after high-choline meals that exceed the recommended daily intake (Zeisel, Growden, et al., 1980). Phosphatidylethanolamine-*N*-methyltransferase (PEMT) rs4646343 and related genotypes were assessed as previously described (Fischer et al., 2010). The details of mass spectroscopy assay are provided in online Supplement S1.

Childhood behavioral measurements and newborn physiology

CBCL1½–5 has 99 items, each scored in a range of 0–2 (absent, sometimes, occurs often) (Achenbach & Rescorla, 2000). Clusters of related behaviors are grouped as syndrome scales (attention, aggression, emotionally reactive, anxious/depressed, sleep, somatic, and withdrawn). The internal reliability ranges from Cronbach's alpha 0.66–0.95. Mothers were asked to complete the CBCL1½–5 when their child was 18, 30, 40, and 48 months old. Electrophysiological recording of cerebral P50 auditory-evoked potentials is provided in online Supplement S1.

Statistical analysis

General linear models and multiple regression were used for analyses. Covariates were established from correlations with the principal outcome, CBCL1½–5 ratings. Effects on Attention Problems in both sexes and Withdrawn scales in males were a priori hypotheses based on the previous clinical trial. Power calculation based on the effect size of choline on CBCL1½–5 Attention Problems in the previous trial, $d' = 0.55$, indicated power $1 - \beta = 0.75$, to observe a similar effect with $N = 82$ and one-third of the mothers having high choline level $\alpha = 0.05$ (one-tail). Two-tail significance is reported for all analyses in this study.

Attrition between enrollment at 16 weeks of gestation and the final assessment 48 months postpartum was assessed based on the choline level. Many mothers missed one or more CBCL1½–5 assessments. Mothers with 16-week gestational choline levels ⩾7.07 μM completed 2.9 (S.D. 1.0) CBCL1½–5 ratings, compared to 3.2 (S.D. 1.0) rating by mothers with lower choline levels, $p = 0.2$. An analysis of variance with repeated measures showed no interaction of the higher or lower choline group with CBCL1½–5 rating time points for Attention Problems, $F_{df1} = 0.002$, $p = 0.964$. Therefore, the ratings for each child were averaged across time points as the principal outcome. Full statistical analyses are provided in online Supplement S2 and eTables S1–S7.

Results

Of the 183 women who consented to the study, 162 brought their newborns for their initial postnatal evaluation at 1 month of age, and 83 continued in the study as their offspring reached early childhood. The principal reason for attrition at each stage was the family moving away from the Denver metropolitan area. The proportion of women with choline concentrations ⩾7.07 μM at the newborn evaluation was 31%; it was 34% at the 40-month evaluation and 25% at the 48-month evaluation. Only one African American woman completed the previous randomized trial. In the current study, 22% who completed were African American or Native American women (Table 1).

Mothers with choline concentrations ⩾7.07 μM were 3 years older on average compared to mothers with lower choline concentrations. There were no other maternal variables or neonatal birth outcomes related to choline concentrations (Table 1). Gestational betaine and dimethylglycine levels did not differ based on the choline level. Maternal body mass index (BMI), prenatal vitamin and folic acid use, which exceeded 90% in both groups, and maternal PEMT genotype also did not differ (Table 1).

Mean maternal choline concentration at 16 weeks of gestation in the group with choline concentration ⩾7.07 μM, 8.47 μM (S.D. 1.56) was significantly lower than the level obtained with phosphatidylcholine supplementation in the randomized trial, 15.21 μM (S.D. 8.14), $t_{df50} = 4.37$, $p < 0.001$. Mean choline levels for the entire group of mothers in the current study rose at 28 weeks of gestation, consistent with other reports of increasing choline concentrations after mid-pregnancy (Orczyk-Pawilowicz et al., 2016; Wu et al., 2012). Maternal plasma choline concentrations at 28 weeks were not associated with outcomes in childhood.

Several maternal covariates were associated with CBCL1½–5 problems scales. They clustered into three groups of intra-associated factors, headed by maternal age, gestational age at birth, and maternal lifetime depression disorder (Table 2).

Offspring of mothers with 16-week gestational choline levels ⩾7.07 μM had fewer problems with attention (both sexes) and

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Table 1. Difference between mothers with higher and lower plasma choline concentrations at 16 weeks of gestation

Parameter	Choline ⩾ 7.07 μM, *N* = 29 (35%)	Choline <7.07 μM, *N* = 54 (65%)	Significance[a]
Maternal characteristics			
Caucasian, *N* (%)	26 (88%)	39 (71%)	
Native American, *N* (%)	1 (4%)	6 (12%)	0.4[b]
African American, *N* (%)	2 (8%)	9 (18%)	0.3[b]
Hispanic ethnicity, *N* (%)	13 (44%)	24 (45%)	>0.9
Maternal age years mean (s.d.)	32.4 (5.2)	29.4 (5.6)	0.03
Maternal education years mean (s.d.)	14.5 (3.2)	14.4 (2.9)	0.9
Living with biological father, *N* (%)	27 (93%)	43 (80%)	0.2
Bipolar disorder DSM-5, *N* (%)	1 (3%)	2 (4%)	>0.9
Depressive disorder DSM-5, *N* (%)	4 (14%)	5 (10%)	0.7
Anxiety disorder DSM-5, *N* (%)	0 (0%)	2 (4%)	0.5
Schizophrenia DSM-5, *N* (%)	0 (0%)	2 (4%)	>0.9
Maternal current smoker, *N* (%)	2 (%)	2 (4%)	0.6
Maternal infection 6–16 weeks of gestation, *N* (%)	10 (33%)	24 (44%)	0.5
Maternal obesity, *N* (%)	10 (36%)	11 (22%)	0.2
Cannabis use at 16 weeks of gestation, *N*	2 (7%)	10 (19%)	0.2
Alcohol use at 16 weeks of gestation, *N* (%)	1 (3%)	7 (13%)	0.3
Adverse childhood experiences mean (s.d.)	2.59 (2.58)	2.51 (2.30)	0.9
Center for Epidemiological Studies of Depression rating 16 weeks of gestation mean (s.d.)	13.6 (8.0)	11.6 (10.8)	0.3
State-Trait Anxiety Inventory-State 16 weeks of gestation mean (s.d.)	33.3 (9.3)	34.6 (12.3)	0.6
Perceived Stress Scale 16 weeks of gestation mean (s.d.)	21.0 (7.2)	23.8 (9.0)	0.2
Center for Epidemiological Studies of Depression rating 6 weeks postpartum mean (s.d.)	8.5 (6.3)	10.9 (9.4)	0.2
State-Trait Anxiety Inventory-State 6 weeks postpartum mean (s.d.)	31.5 (9.2)	32.3 (10.0)	0.7
Perceived Stress Scale 6 weeks of postpartum mean (s.d.)	19.2 (7.6)	21.4 (8.4)	0.2
Parenting Stress Inventory mean (s.d.)	24.9 (7.5)	24.1 (9.1)	0.7
Labor and delivery			
Pre-eclampsia, *N* (%)	3 (12%)	7 (12%)	>0.9
Gestational diabetes, *N* (%)	1 (4%)	4 (8%)	0.7
<33 weeks of gestation, *N* (%)	0 (0%)	5 (10%)	0.2
Vaginal delivery, *N* (%)	22 (77%)	40 (68%)	0.6
Neonatal characteristics			
Gestational age at birth days mean (s.d.)	273 (12)	274 (16)	0.7
APGAR 5 min mean (s.d.)	8.62 (1.17)	8.76 (0.60)	0.5
Birthweight (g) mean (s.d.)	3163 (455)	3140 (563)	0.8
Comparison of maternal metabolic parameters			
Maternal plasma levels 16 weeks of gestation mean (s.d.)			
Choline (μM)	8.47 (1.56)	5.38 (1.05)	<0.001[a]
Betaine (μM)	10.6 (2.9)	11.5 (3.2)	0.2[a]
Dimethylglycine (μM)	9.01 (3.32)	9.00 (3.21)	>0.9[a]
Maternal plasma levels 28 weeks of gestation mean (s.d.)			
Choline (μM)	7.40 (1.86)	7.16 (1.98)	0.6[a]
Betaine (μM)	10.5 (3.2)	11.1 (2.5)	0.4[a]

(*Continued*)

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Table 1. (*Continued.*)

Parameter	Choline ⩾ 7.07 μM, *N* = 29 (35%)	Choline <7.07 μM, *N* = 54 (65%)	Significance[a]
Dimethylglycine (μM)	7.84 (2.09)	7.21 (1.97)	0.2[a]
Factors potentially influencing maternal choline level, *N* (%)			
Prenatal vitamins with folic acid	27 (93%)	51 (94%)	>0.9[b]
Pre-pregnancy BMI	26.4 (5.5)	27.7 (7.9)	0.5[b]
Term BMI	31.1 (4.8)	32.9 (7.9)	0.3[b]
PEMT rs4646343			
GG	9 (41%)	15 (44%)	$\chi^2_{df2} = 3.62$, $p = 0.2$
GT	8 (36%)	17 (50%)	
TT	5 (23%)	2 (6%)	

[a]Student's *t* test or Fisher's exact test.
[b]Compared to Caucasian women.

Table 2. Co-variates associated with CBCL1½–5 Attention problems

	CBCL1½–5, Attention Problems *T* score	Maternal age (years)	Gestational age at birth	Lifetime DSM-5 Depression
Maternal age (years)	−0.222*			−0.0043
Gestational age at birth	0.224*	0.015		0.064
African American or Native American	0.062	−0.261*	−0.321**	−0.096
Hispanic ethnicity	0.046	−0.110*	0.098	0.136
Maternal education (years)	0.054	0.220*	0.130	−0.111
Adverse childhood experiences	0.184	0.100	−0.124	0.072
Lifetime DSM-5 Depression	0.324**	−0.043	0.139	
Maternal infection 16 weeks of gestation	0.265*	−0.300***	0.104	0.184
Cannabis use 16 weeks of gestation	0.475***	−0.107	0.183	0.269*

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

social withdrawal (males) behaviors on the CBCL1½–5 compared to children of mothers with lower plasma choline concentrations (choline level, Wald $\chi^2_{df1} = 3.837$, $p = 0.050$; Table 3, online Supplementary Table e1). Children whose mothers had higher plasma choline concentrations had lower Attention Problem *T*-scores of 52.8, S.E. 1.0 (percentile 58.5, S.E. 2.4) compared to children whose mothers had lower choline concentrations, Attention Problem *T*-score, percentile 55.2, S.E. 0.2 (percentile 65.3, S.E. 1.8); $p = 0.050$. For male children, Withdrawn *T*-scores were also lower if mothers had higher choline concentrations, *T*-score 52.0, S.E. 1.2 (percentile 59.2, S.E. 3.2), *v.* *T*-score 57.6, S.E. 1.2 (percentile 68.3, S.E. 2.5); $p = 0.007$, for children whose mothers had lower choline concentrations (choline level × child sex, Wald $\chi^2_{df1} = 5.362$, $p = 0.021$; Fig. 1; online Supplementary Table e2). Higher choline concentrations were also associated with fewer Sleep Problems, *T*-score 52.1, S.E. 1.1 (percentile 58.3, S.E. 2.2), compared to *T*-score 55.0, S.E. 0.8 (percentile 62.2, S.E. 1.6); $p = 0.037$, for children of mothers with lower choline concentrations (choline level, Wald $\chi^2_{df1} = 4.354$, $p = 0.037$, online Supplementary Table e3).

As was found in the clinical trial of phosphatidylcholine supplementation, maternal choline concentrations at 16 weeks of gestation were associated with increased inhibition of the P50 cerebral auditory-evoked potential, and poorer fetal development of this cerebral inhibition was associated with increased Attention Problems in childhood (Ross et al., 2016). The correlation of choline concentration with increased newborn P50 cerebral-evoked potential amplitudes to the second of paired auditory stimuli, indicating poorly developed cerebral inhibition, was $\beta = -0.122$, $p = 0.048$ (online Supplementary Table e4). The correlation of increased newborn P50 cerebral-evoked potential amplitudes to the second of paired auditory stimuli, indicating poorly developed cerebral inhibition, to higher CBCL1½–5 Attention Problem *T*-scores, was $\beta = 0.381$, $p = 0.003$ (online Supplementary Table e5).

Both prenatal cannabis and common maternal viral and bacterial infections were associated with increased CBCL1½–5 syndrome scale scores, including Attention Problems (Table 4). Higher maternal choline concentrations were associated with decreased Attention Problems scores in children of mothers who used cannabis or had infections (choline level, Wald $\chi^2_{df1} = 5.367$, $p = 0.021$, Table 5, online Supplementary Table e6). For children of mothers who used cannabis in gestation, Attention Problems scores were significantly lower if the mother also had higher choline concentrations, *T*-score 53.1, S.E. 3.4 (percentile 60.3, S.E. 8.4), than if the mother had lower choline concentrations,

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Table 3. Relation of maternal choline plasma concentration at 16 weeks of gestation to childhood behavior problems

CBCL1½–5, 18–48 months marginal mean (s.e.)	Maternal choline ⩾7.07 μM	Maternal choline <7.07 μM	Significance *p* =
Attention *T*-score	52.8 (1.0)	55.2 (0.2)	0.050
Attention percentile	58.5 (2.4)	65.3 (1.8)	
Social withdrawal male *T*-score	52.0 (1.2)	57.6 (1.2)	0.007
Socially withdrawn male percentile	59.2 (3.2)	68.3 (2.5)	
Socially withdrawn female *T*-score	54.5 (1.7)	53.2 (1.2)	0.5
Social withdrawal female percentile	59.5 (3.2)	59.4 (2.2)	
Sleep *T*-score	52.1 (1.1)	55.0 (0.8)	0.04
Sleep percentile	58.3 (2.3)	62.2 (1.6)	
Anxious depressed *T*-score	52.6 (0.8)	53.4 (0.6)	0.4
Anxious depressed percentile	58.4 (2.1)	60.5 (1.5)	
Emotionally reactive *T*-score	53.0 (0.9)	53.5 (0.7)	0.6
Emotionally reactive percentile	59.6 (2.2)	58.6 (1.6)	
Somatic *T*-score	53.5 (0.8)	53.7 (0.6)	0.8
Somatic percentile	60.7 (2.2)	61.1 (1.6)	
Aggression *T*-score	54.8 (1.1)	53.3 (0.9)	0.3
Aggression percentile	60.0 (2.3)	59.9 (1.7)	
Total problems *T*-score	45.5 (1.8)	48.1 (1.3)	0.3
Total problems percentile	35.9 (4.9)	43.3 (3.6)	

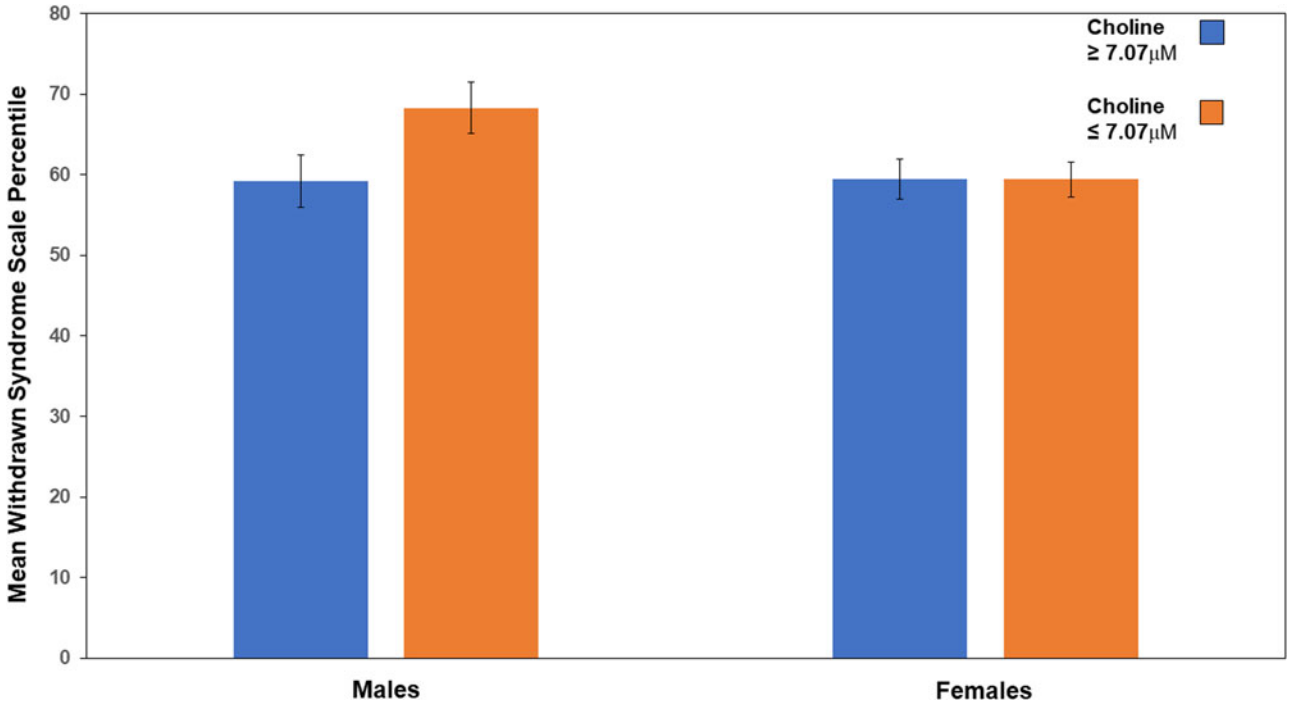

Fig. 1. Mean percentiles for scores on the CBCL1½–5 Withdrawn Syndrome Scale shown separately for males and females by maternal choline concentrations. Scores were significantly lower for male children of mothers with higher choline concentrations ($p = 0.007$).

T-score 61.1, s.e. 1.6 (percentile 76.4, 4.0); $p = 0.034$. For children of mothers who had gestational infections, Attention Problems scores were also lower if the mother also had higher choline concentrations, *T*-score 53.5, s.e. 2.0 (percentile 60.9, s.e. 5.0) than if the mother had lower choline concentrations, *T*-score 58.0, s.e. 1.1 (percentile 69.9, s.d. 2.8; $p = 0.050$) (Fig. 2).

CBCL1½–5 includes scales that group problems into DSM-5 categories. Ratings ⩾92nd percentile on the Attention Deficit

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Table 4. Effects of maternal cannabis and infection at 16 weeks of gestation on Child Behavioral Checklist/1½–5 problems

CBCL1½–5 *T*-score mean (s.d.)	Maternal cannabis, *N* = 12	No cannabis, *N* = 71	Maternal infection, *N* = 30	No infection, *N* = 53
Attention	61.2 (9.9)*	53.3 (4.0)	56.5 (7.8)*	53.2 (4.1)
Social withdrawn	60.2 (11.2)*	53.6 (5.3)	55.4 (6.9)	54.2 (6.8)
Sleep	62.6 (10.1)**	52.6 (4.3)	56.2 (8.0)*	52.8 (5.2)
Anxiety depression	57.5 (6.5)*	52.4 (3.7)	54.8 (5.2)	52.1 (3.8)
Emotionally reactive	59.3 (8.9)*	52.3 (4.1)	54.7 (6.7)	52.6 (4.7)
Somatic	58.2 (5.7)**	52.9 (4.0)	54.4 (4.7)	53.2 (4.6)
Aggressive	61.2 (10.9)*	52.6 (4.8)	56.7 (9.7)	52.3 (3.5)
Total problems	60.1 (10.8)***	45.0 (9.4)	50.0 (12.5)*	45.1 (9.4)

*Students *t* test: $p < 0.05$, **$p < 0.01$, *$p < 0.001$.

Table 5. Relation of choline plasma concentration at 16 weeks of gestation and gestational cannabis use or maternal infection with childhood attention problems

CBCL1½–5 Attention problems 18–48 months *T*-score, percentile marginal mean (s.e.)	Cannabis 16 weeks of gestation	No Cannabis 16 weeks of gestation	
(A) Gestational cannabis use			
Choline ⩾7.07 μM	53.1 *T*-score (3.4) 60.3 percentile (8.4)	53.0 *T*-score (1.0) 59.9 percentile (2.5)	$p = 0.97$
Choline <7.07 μM	61.1 *T*-score (1.6) 76.4 percentile (4.0)	53.9 *T*-score (0.8) 62.1 percentile (1.9)	$p < 0.001$
	$p = 0.034$	$p = 0.48$	
CBCL1½–5 Attention problems 18–48 months percentile	Maternal infection 16 weeks of gestation	No infection 16 weeks of gestation	
(B) Maternal infection			
Choline ⩾7.07 μM	53.5 *T*-score (2.0) 60.9 percentile (5.0)	52.6 *T*-score (1.9) 59.3 percentile (4.0)	$p = 0.65$
Choline <7.07 μM	58.0 *T*-score (1.1) 69.9 percentile (2.8)	57.1 *T*-score (1.1) 68.7 percentile (2.8)	$P = 0.53$
	$p = 0.050$	$p = 0.046$	

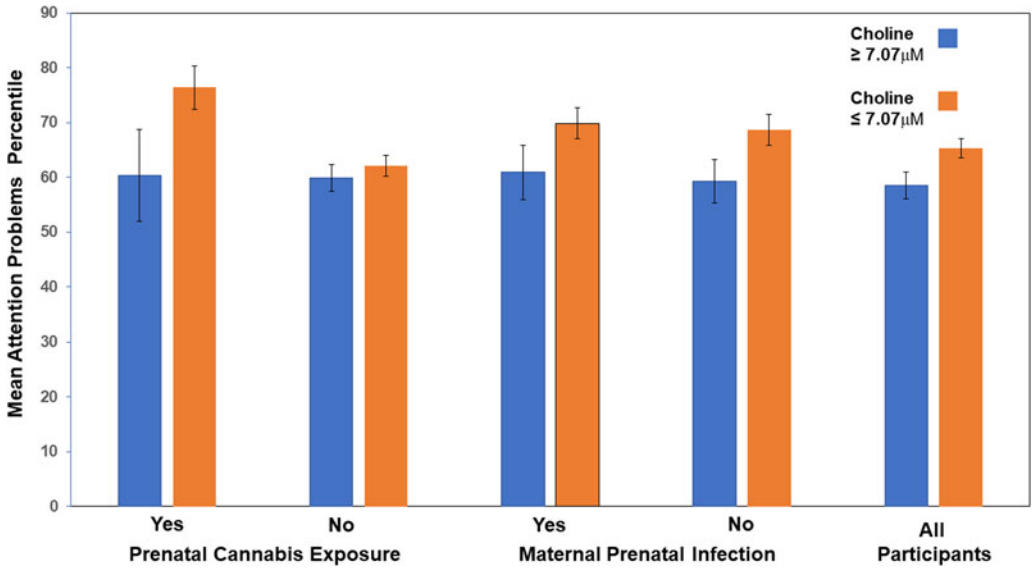

Fig. 2. Mean percentiles for scores on the CBCL1½–5 Attention Problems Scale shown separately for prenatal cannabis exposure, maternal prenatal infection, and for all participants by maternal choline concentrations. Scores are lower for children whose mothers had higher choline concentrations (all participants, $p = 0.050$). This relationship was also true for children with prenatal cannabis exposure ($p = 0.034$) as well as children whose mothers who experienced infection during gestation ($P = 0.050$).

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Hyperactivity Disorder (ADHD) scale occurred in 8 of the 83 children between ages 40 and 48 months. Ratings at this level are generally associated with children who present for clinical evaluations on the referral of parents, schools, and physicians, according to the CBCL1½–5 manual (Achenbach & Rescorla, 2000). Seven of these eight children were from mothers who had lower choline concentrations.

Discussion

Increased maternal plasma choline concentrations in the early second trimester of pregnancy were associated with decreased problems in attention in both sexes and withdrawal in male children on the widely used Child Behavior Checklist/1½–5 years. These results are similar to those obtained in the earlier double-blind, placebo-controlled trial of phosphatidylcholine supplementation beginning in the second trimester. In the current study, we found higher maternal choline levels were associated with decreased adverse effects of maternal cannabis use and infection. Other prenatal nutrients, notably folic acid and vitamin D, have also been associated with cognitive and behavioral benefits for the offspring (McGrath et al., 2010; Roza et al., 2010). Their use by nearly 95% of mothers in this study obviated assessment of their effects.

The association of choline concentrations at 16 weeks of gestation with cerebral P50 auditory-evoked potential inhibition in newborns and the relationship of the inhibition to childhood attention problems, found in both studies, are consistent with long-term behavioral effects of choline on fetal interneuron development. As we found in this study with children, P50 inhibition is related to attention in both patients with schizophrenia and in the general population (Hamilton et al., 2018; Wan, Friedman, Boutros, & Crawford, 2008).

The mean level of maternal choline concentration reached in this study was just over half the level reached with phosphatidylcholine supplementation in previous randomized trials (Cheatham et al., 2012; Ross et al., 2016). Choline is concentrated in the amniotic fluid by active transporters in the placenta, and therefore fetal concentrations are unknown (Baumgartner et al., 2015). The levels in the current study were sufficient to be associated with significantly decreased effects of cannabis and infection. Studies of childhood outcome with various risk factors, comparing dose and timing of choline supplements as well as maternal plasma levels, will be necessary to establish optimal levels. The most recent FDA advisory raised the amount to 550 mg (Food and Drug Administration, 2016). Choline plasma concentration 7.0 μL at 16 weeks of gestation has been estimated to be a level reflecting a diet meeting minimum requirements (Wu et al., 2012). The 35% of women with choline levels ⩾7.07 μM in this study is consistent with other studies finding that only about one-third of women have either adequate levels or sufficient dietary intake (Jensen, Batres-Marquez, Carriquiry, & Schalinske, 2007; Masih et al., 2015; Wu et al., 2012). Choline concentrations are lower in the first half of pregnancy and then rise as pregnancy progresses to term (Orczyk-Pawilowicz et al., 2016; Wu et al., 2012). The 28-week concentrations were not related to childhood outcomes in this study, although two trials with supplements have found them helpful in the last half of pregnancy. The absence of relation of choline concentrations to betaine and dimethylglycine concentrations indicates that most of the choline is being consumed to synthesize phosphatidylcholine for fetal and placental membranes (Zeisel, 2006a). The remaining level may then be too low to fully activate α7-nicotinic acetylcholine receptors in some pregnancies.

The 4-year results are the longest-term outcomes observed from a clinical supplementation trial or cohort study based on plasma choline levels. Differences in childhood outcomes based on the estimates of choline from dietary intake have been observed for up to 7 years of age (Boeke et al., 2013). Longer-term studies are desirable because the outcomes approach clinically meaningful endpoints, such as childhood attention deficit disorder. However, the 4.5 years between enrollment in the first trimester and final childhood study were accompanied by high attrition rates. Although attrition was not related to the choline level, it is a limitation of the study. A second limitation of longer-term postnatal follow-up is that postnatal and prenatal effects cannot be rigorously distinguished. Newborn P50 inhibition was associated with childhood outcomes 4 years later, consistent with the influence of early prenatal choline effects despite postnatal effects of maternal rearing. However, the possibility that maternal or other variables account for both choline concentration and outcome cannot be ruled out in an observational study, unlike in the randomized supplementation trial. A third limitation is that we did not obtain polygenic risk scores for schizophrenia or attention deficit disorder for the children, which are associated with neurodevelopmental problems (Riglin et al., 2017). Nor do we have polygenic risk scores for the mothers, which are associated with both their likelihood of infection and the genetic transmission of risk for neurodevelopmental illnesses (Leppert et al., 2019). A fourth limitation is that the higher choline concentrations in this cohort did not alleviate all the effects of gestational cannabis use or infection. The decrease in attention problems associated with higher choline levels would be expected to be meaningful as the child develops, but other effects of cannabis and infection were present at 4 years of age that were not mitigated by higher choline concentrations, prenatal vitamins with folic acid, and the prenatal and obstetrical care that the women received. Finally, the sample size ($N = 12$) for the maternal cannabis group was small. These data should be interpreted with caution, and replication in a larger sample is necessary.

This study extends previous studies in several ways. It provides a second assessment of higher gestational choline concentrations with 4-year outcome on a broad range of behavioral measures on a widely used clinical scale. The results are consistent with the previous randomized clinical trial. A more diverse maternal population was studied. In addition, the current study assesses choline levels in the context of a wider range of maternal risk factors, notably infection and cannabis use, than were possible in the randomized trial because of FDA restrictions to healthy women in that trial's Investigational New Drug Application (IND). Both maternal infection from the current COVID-19 epidemic and increasing rates of maternal cannabis use are likely to impact child development and the risk for mental illness in the next decades (Centers for Disease Control and Prevention, 2020; Roncero et al., 2020; Volkow, Han, Compton, & McCance-Katz, 2019). This study detected the effects of prenatal infection and cannabis use in childhood and the association of higher choline levels with their decreased effects on childhood attention. Problems in attention and social withdrawal are not only disabling in childhood, but they also are associated with emergence of mental illness in early adulthood, including schizophrenia (Cassidy, Joober, King, & Malla, 2011; Matheson et al., 2013; Rossi, Pollice, Daneluzzo, Marinangeli, & Stratta, 2000). Higher maternal choline concentrations

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are associated with decreased problems in attention and social withdrawal. Unfortunately, results from several studies suggest the majority of women do not obtain adequate intake levels of choline from their diet and may require supplements to reach optimal concentration levels to protect fetal brain development and subsequent childhood behavior (Jensen et al., 2007; Masih et al., 2015; Ross et al., 2016).

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0033291720005061

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Conflict of interest. The authors report no conflicts of interest.

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