

Polymorphisms in *SLC44A1* are associated with cognitive improvement in children diagnosed with fetal alcohol spectrum disorder: an exploratory study of oral choline supplementation

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ABSTRACT

Background: The essential nutrient choline provides one-carbon units for metabolite synthesis and epigenetic regulation in tissues including brain. Dietary choline intake is often inadequate, and higher intakes are associated with improved cognitive function.

Objective: Choline supplements confer cognitive improvement for those diagnosed with fetal alcohol spectrum disorder (FASD), a common set of neurodevelopmental impairments; however, the effect sizes have been modest. In this retrospective analysis, we report that genetic polymorphisms affecting choline utilization are associated with cognitive improvement following choline intervention.

Methods: Fifty-two children from the upper midwestern United States and diagnosed with FASD, ages 2–5 y, were randomly assigned to receive choline (500 mg/d; $n = 26$) or placebo ($n = 26$) for 9 mo, and were genotyped for 384 choline-related single nucleotide polymorphisms (SNPs). Memory and cognition were assessed at enrollment, study terminus, and at 4-y follow-up for a subset.

Results: When stratified by intervention (choline vs. placebo), 14–16 SNPs within the cellular choline transporter gene solute carrier family 44 member 1 (*SLC44A1*) were significantly associated with performance in an elicited imitation sequential memory task, wherein the effect alleles were associated with the greatest pre/postintervention improvement. Of these, rs3199966 is a structural variant (S644A) and rs2771040 is a single-nucleotide variant within the 3' untranslated region of the plasma membrane isoform. An additive genetic model best explained the genotype associations. Lesser associations were observed for cognitive outcome and polymorphisms in flavin monooxygenase-3 (*FMO3*), methylenetetrahydrofolate dehydrogenase-1 (*MTHFD1*), fatty acid desaturase-2 (*FADS2*), and adiponectin receptor 1 (*ADIPOR1*).

Conclusions: These *SLC44A1* variants were previously associated with greater vulnerability to choline deficiency. Our data potentially support the use of choline supplements to improve cognitive function in individuals diagnosed with FASD who carry these effect alleles. Although these findings require replication in both retrospective and prospective confirmatory trials, they emphasize

the need to incorporate similar genetic analyses of choline-related polymorphisms in other FASD-choline trials, and to test for similar associations within the general FASD population. This trial was registered at www.clinicaltrials.gov as NCT01149538. *Am J Clin Nutr* 2021;114:617–627.

Keywords: fetal alcohol spectrum disorder, choline, *SLC44A1*, precision nutrition, single nucleotide polymorphism (SNP), rs3199966, rs2771040, nutrigenomics

Introduction

The essential nutrient choline provides one-carbon units for numerous biochemical reactions and DNA and histone methylation (1, 2). In the brain, it contributes to acetylcholine synthesis and cholinergic function, myelination, and the major lipids phosphatidylcholine and sphingomyelin (3). Dietary choline intake is often limiting during the high-demand periods of pregnancy and lactation (4, 5), and higher intakes during pregnancy and

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Supplemental Figure 1 and Supplemental Tables 1–3 are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents at <https://academic.oup.com/ajcn/>.

Abbreviations used: CBCL, Child Behavior Checklist; *FADS2*, fatty acid desaturase-2; FASD, fetal alcohol spectrum disorder; *FMO3*, flavin monooxygenase-3; *MTHFD1*, methylenetetrahydrofolate dehydrogenase-1; PCA, principal components analysis; SB-5, Stanford-Binet Intelligence Scale, version 5; SNP, single nucleotide polymorphism; *SLC44A1*, solute carrier family 44 member 1; 3'-UTR, 3'-untranslated region.

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early childhood are associated with improved memory, cognition, and attention (5–9) and confer cognitive protection in preclinical models of Down syndrome, Rett syndrome, autism spectrum disorder, and iron deficiency (5, 6, 10). Mechanistically, choline promotes hippocampal and cortical neurogenesis, cholinergic maturation and function, and neuronal survival, in part through effects on DNA methylation and gene expression (5, 6, 11). These actions stimulated interest in choline's potential to remediate the cognitive, learning, and memory deficits that typify fetal alcohol spectrum disorder (FASD), the clinical sequelae of prenatal alcohol exposure (12, 13). Three to five percent of US first-graders meet criteria for an FASD diagnosis (14), and there is high interest in interventions that mitigate alcohol's damage.

Although preclinical models of FASD consistently report that supplemental choline improves cognition, (15), outcomes from clinical intervention trials are more nuanced. In young children diagnosed with FASD, choline (500 mg/d for 9 mo) did not improve pre/post measures of global cognitive functioning (16, 17); however, the youngest children (2.5–4.0 y) had significant improvement in the delayed memory component of a hippocampal-dependent task. In a subset who returned for a 4-y follow-up (18), the choline group had increased nonverbal visual spatial reasoning ($P = 0.004$) and working memory ($P = 0.018$), nonverbal IQ ($P = 0.03$), and working memory ($P = 0.01$) relative to placebo. In a gestational intervention, infants exposed prenatally to alcohol who received 740 mg choline/d (19) had significantly improved visual memory scores in a habituation-dishabituation test (20) but did not differ from placebo controls on the Bayley Scales of Infant Development (21). Another trial that used a higher choline dose (2 g/d) found that maternal adherence to the choline intervention predicted behavioral improvement ($r = 0.63$, $P < 0.01$) (21), and those infants performed better in an eyeblink conditioning task ($P < 0.01$) at age 6 mo and a visual recognition memory task at age 12 mo.

We hypothesized that genetic differences in choline utilization may partially explain these improvements. Polymorphisms in multiple choline-related genes, including *BHMT*, *CHDH*, *CHKA*, *CHDH*, flavin monooxygenase-3 (*FMO3*), methylenetetrahydrofolate dehydrogenase-1 (*MTHFD1*), *PEMT*, and *SLC44A1*, operate as effect alleles that reduce choline synthesis, utilization, or absorption, and thus increase individual choline needs, particularly when intake is limiting (22–30). In this exploratory study, we identify multiple polymorphisms within the choline transporter gene *SLC44A1* that associate with cognitive improvement in response to oral choline supplementation, in children diagnosed with FASD.

Methods

Study design and participants

This retrospective analysis used data from a pilot trial wherein 65 individuals diagnosed with FASD, ages 2–5 y and recruited from across the upper midwestern United States, were randomly assigned in a double-blinded trial (NCT01149538) to receive a daily intervention of either 500 mg choline ($n = 31$), administered as 1.25 g choline bitartrate in a flavored beverage, or the same beverage but lacking choline (placebo; $n = 29$). The study design (Figure 1) and methods are detailed elsewhere (16–18). This choline dose is twice the Adequate Intake and was

selected to be well tolerated and achievable through dietary means (16). Participants received the intervention for 9 mo. The study was completed in 2 phases. The primary outcomes of phase 1 was to assess the side effects or adverse events to the choline intervention; these data were reported in Wozniak et al. (16). For the second phase, the primary outcome was an exploratory assessment of global cognitive functioning, using the Mullen Scales of Early Learning, at baseline and 9 mo (17), and the initial sample size was set to detect an effect size of 0.43 on this measure. The secondary outcome was exploratory assessment of hippocampal-dependent memory function, using an elicited imitation memory paradigm, testing both immediate and delayed recall, at baseline, 6 mo, and 9 mo (17). In a follow-up study 4 y later, a subset of 31 participants (16 placebo, 15 choline) returned for follow-up assessment that included the Stanford-Binet Intelligence Scale, version 5 (SB-5), an age-appropriate elicited imitation memory paradigm; 3 subtests within a developmental NEUROPSYCHOLOGICAL Assessment, second edition (NEPSY-II: Memory for Names, Memory for Faces, Narrative Memory); tests of executive function from the NIH Toolbox; and the parent-report Child Behavior Checklist (18). Serum choline, betaine, and phosphatidylcholine were quantified at baseline (0 mo) and at 6 mo and 9 mo 3 h after administering the choline or placebo, using LC/electrospray ionization–isotope dilution MS, as described (17). All procedures were in accordance with the ethical standards of the University of Minnesota's Human Research Protection Program, and in accordance with the Helsinki Declaration of 1975 as revised in 1983.

Single nucleotide polymorphism analysis

At the time of the original study, genomic DNA was extracted from blood samples of 52 participants (26 choline, 26 placebo); blood samples were not obtained from 5 participants in the choline group and 3 in the placebo group due to child refusal, difficult veins, etc. Single nucleotide polymorphism (SNP) analysis was performed using an oligo-specific extension-ligation assay on a custom Golden Gate array (Illumina) that contained 384 SNPs in genes relevant to choline and one-carbon metabolism (30), and selected based on published identification of SNPs that altered choline status or affected choline metabolism (24, 25, 29, 31). The data processing and analysis of SNP associations were performed in R (version 3.6.3; R Foundation for Statistical Computing; <http://cran.r-project.org/>). Using the Entrez global query cross-database search system, 365 SNP identities were mapped into 40 choline-related genes or loci using Rentrez package (version 1.2.2); 19 SNPs could no longer be matched but were retained for further analysis. SNPs removed from analysis were those having a homozygous distribution ($n = 60$), a frequency of <0.05 ($n = 51$), or that violated Hardy-Weinberg equilibrium of random mating ($n = 24$). Thus, the final analysis interrogated 243 SNPs (Supplemental Table 1). Principal components analysis (PCA) from the FactoMineR package (version 2.3; Comprehensive R-Archive Network) was used to examine the genomic separation of participants based on age, sex, race, and treatment group.

The phenotype data set consisted of 230 physical and behavioral characteristics, of which 25 were categorical. The behavioral traits (Table 1) included cognitive performance at baseline and at the end of choline intervention ($n = 26$ choline,

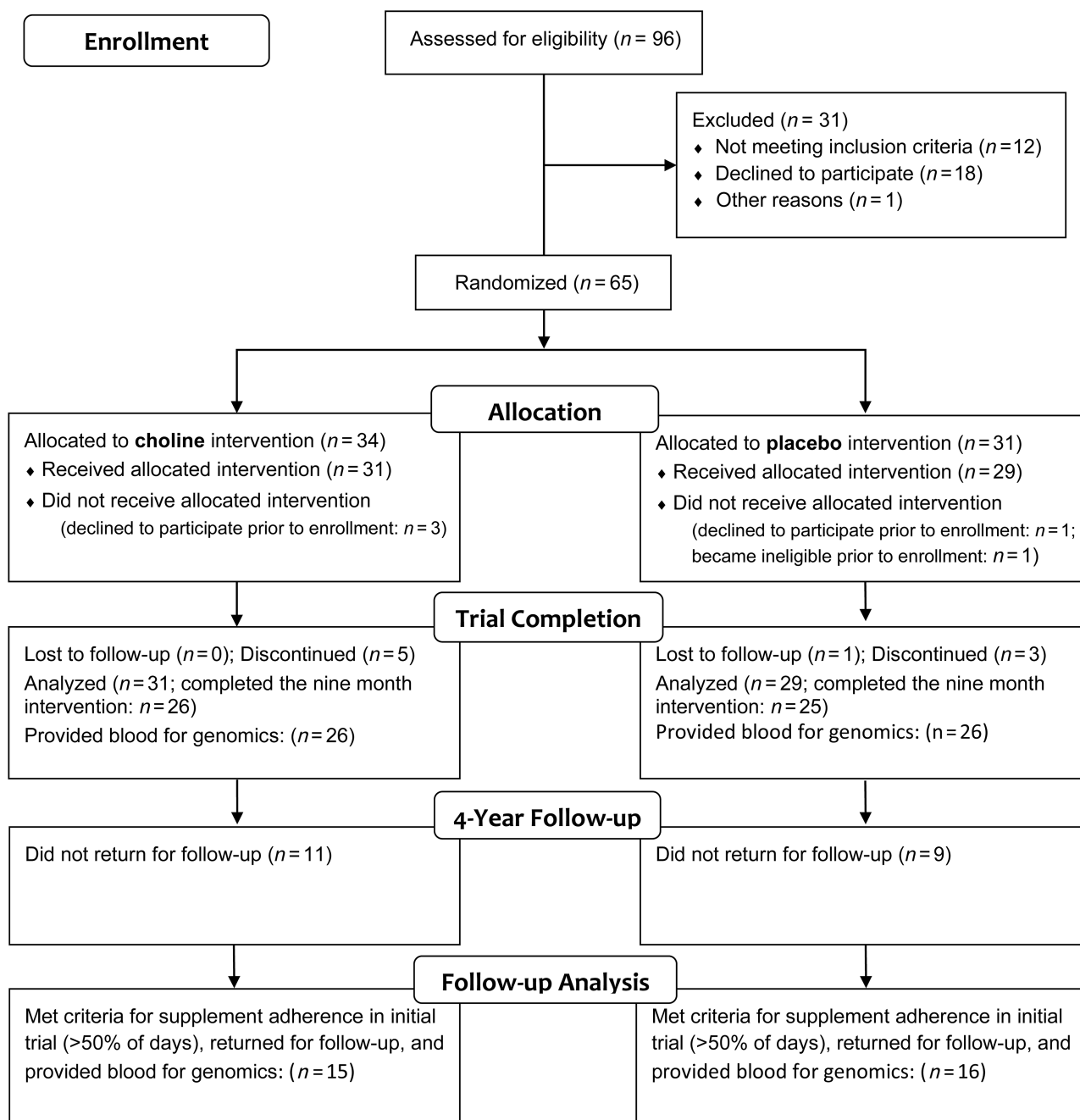


FIGURE 1 CONSORT flow diagram of the randomized postnatal choline intervention trial. CONSORT, Consolidated Standards of Reporting Trials.

$n = 26$ placebo) and at the 4-y follow-up ($n = 15$ choline, $n = 16$ placebo). Some variables were missing for some participants and, in these instances, participants were nonetheless included for their other existing data. We performed separate association tests between the 243 SNPs and the 230 phenotype categories, and tested for potential interactions under Additive, Dominant, and Recessive genetic models using the SNPAssoc package (version 1.9–2) (32). For all 3 genetic models, simple linear modeling was used to apply logistic regression for categorical phenotypes and linear regression for numerical phenotypes; the base R package stats (version 3.6.3) was used to extract P values, coefficients, and

effect alleles. P values were adjusted for multiple testing using Bonferroni correction, accounting for SNPs that were in perfect linkage disequilibrium, and a Q value ≤ 0.05 was considered significant.

Results

There were no differences in the characteristics of these genotyped participants at baseline and at the 4-y follow-up (Table 2) (16–18). Average daily dietary choline intake did

TABLE 1 Definitions of outcome measures¹

	Definitions
activity_level.11	Parent-reported child activity level from the CBCL (short form) at 9 mo; range = 1 to 7, higher scores indicate higher levels of activity
blood_pressure_diastolic.1	Diastolic blood pressure at baseline (0 mo)
blood_pressure_diastolic.8	Diastolic blood pressure at 6 mo
bloodlevel_betaine.1	Plasma betaine concentrations at baseline (0 mo)
delta_adjpairs_imm	Change in Elicited Imitation immediate memory task performance (for pairs of items regardless of sequence order) between baseline (0 mo) and 9 mo; higher scores indicate greater positive change (improvement)
delta_imt_v1_v13_adjpairs	Change in Elicited Imitation memory task performance (for pairs of adjacent items in the correct sequence order) between baseline (0 mo) and 4-y follow-up; higher scores indicate greater positive change (improvement)
delta_pairs_imm	Change in Elicited Imitation immediate memory task performance (pairs) between baseline (0 mo) and 9 mo; higher scores indicate greater positive change (improvement)
heart_rate.11	Resting heart rate at 9 mo
height_physical.11	Height taken during physical examination at 9 mo
height_pile_physical.1	Age-adjusted height percentile at baseline (0 mo)
height_pile_physical.11	Age-adjusted height percentile at 9 mo
height_z_physical.1	z Score for age-adjusted height at 0 mo
height_z_physical.11	z Score for age-adjusted height at 9 mo
ibb_tscore21	CBCL parent-reported “sluggish cognitive tempo” at baseline (0 mo); age-corrected scores with higher scores indicating more problems
iib_avg_imt_adjpairs	Elicited Imitation memory task performance (adjacent pairs) at 4-y follow-up; higher scores indicate better memory performance
iib_tscore13	CBCL parent-reported Externalizing problems at baseline (0 mo); age-corrected scores with higher scores indicating more problems
iib_tscore21	CBCL parent-reported Conduct problems at baseline (0 mo); age-corrected scores with higher scores indicating more problems
ilb_avg_imt_pairs	Elicited Imitation memory task performance (pairs) at 4-y follow-up; higher scores indicate better memory performance
imt_adjpairs_imm_med.11	Elicited Imitation immediate memory task performance (adjacent pairs) at 9 mo; higher scores indicate better memory performance
imt_comp_imm_med.1	Elicited Imitation immediate memory task performance (components) at baseline (0 mo); higher scores indicate better memory performance
imt_comp_sd_avemh.11	Elicited Imitation short-delay memory task performance (components) at 9 mo; higher scores indicate better memory performance
memfaces_delcontrastsc	NEPSY-II Memory for Faces—Immediate vs. Delayed contrast score at 4-y follow-up; age-adjusted scaled score with higher scores indicating better performance
memnames_t3total	NEPSY-II Memory for Names Trial 3 total score at 4-y follow-up; age-corrected scaled score with higher scores indicating better performance
nihdccs_agecorrstandsc	NIH Toolbox Dimensional Card Sorting test performance at 4-y follow-up; age-corrected standard score with higher scores indicating better performance
nihdccs_fullcortsc	NIH Toolbox Dimensional Card Sort Test performance at 4-y follow-up; fully corrected (age, education, gender, race/ethnicity) standard score with higher scores indicating better performance
nihpsmt_agecorrstandsc	NIH Toolbox Picture Sequence Memory Test performance at 4-y follow-up; age-corrected standard score with higher scores indicating better performance
stanfbi_fsiq	Stanford-Binet Intelligence Scale (5th edition) Full Scale Intelligence Quotient at 4-y follow-up; age-corrected scores with higher scores indicating higher ability
stanfbi_qr	Stanford-Binet Intelligence Scale (5th edition) Quantitative Reasoning Index at 4-y follow-up; age-corrected scores with higher scores indicating higher ability
stanfbi_viq	Stanford-Binet Intelligence Scale (5th edition) Verbal Intelligence Quotient at 4-y follow-up; age-corrected scores with higher scores indicating higher ability
stanfbi_wm	Stanford-Binet Intelligence Scale (5th edition) Working Memory index at 4-y follow-up; age-corrected scores with higher scores indicating higher ability
t_score_2.11	CBCL parent-reported social problems at 9 mo; age-corrected scores with higher scores indicating more problems
t_score3.1	CBCL parent-reported school problems at baseline (0 mo); age-corrected scores with higher scores indicating more problems
t_score8.11	CBCL parent-reported thought problems at 9 mo; age-corrected scores with higher scores indicating more problems
weight_pile_physical.1 ²	Age-adjusted weight percentile at 0 mo
weight_pile_physical.3 ²	Age-adjusted weight percentile at 3 mo
weight_z_physical.1 ²	z Score for age-adjusted height at 0 mo
weight_z_physical.3 ²	z Score for age-adjusted height at 3 mo
weight_z_physical.8 ²	z Score for age-adjusted height at 6 mo
weight_z_physical.11 ²	z Score for age-adjusted height at 9 mo
weightheight_z_physical.1 ²	z Score for age-adjusted weight-height at 0 mo

¹Child Behavior Checklist; NEPSY-II, NEuroPSYcological Assessment, second edition.²Height and weight were standardized using normative data (33).

TABLE 2 Characteristics of participants¹

	Participants at baseline		Participants at 4-y follow-up	
	Placebo (<i>n</i> = 26)	Choline (<i>n</i> = 26)	Placebo (<i>n</i> = 16)	Choline (<i>n</i> = 15)
Age at enrollment, y	3.88 ± 0.78	3.84 ± 0.78	3.81 ± 0.78	3.92 ± 0.74
Age at follow-up, y	—	—	8.44 ± 1.06	8.61 ± 0.99
Years since study completion	—	—	3.96 ± 0.60	3.96 ± 0.46
Sex, <i>n</i> (%)				
Male	9 (34.6)	10 (38.5)	7 (43.8)	8 (53.3)
Female	17 (65.4)	16 (61.5)	9 (56.2)	7 (46.7)
Racial categories, ² <i>n</i> (%)				
White	7 (26.9)	12 (46.2)	4 (25.0)	7 (46.7)
Black or African American	9 (34.6)	4 (15.4)	5 (31.2)	2 (13.3)
American Indian/Alaska Native	6 (23.0)	4 (15.4)	4 (25.0)	2 (13.3)
Asian	1 (3.8)	1 (3.8)	1 (6.3)	1 (6.7)
More than 1 race	3 (11.5)	4 (15.4)	2 (12.5)	2 (13.3)
Not reported	0 (0)	1 (3.8)	0 (0)	1 (6.7)
Ethnic category, ² <i>n</i> (%)				
Hispanic or Latino	1 (3.8)	1 (3.8)	1 (6.3)	1 (6.7)
Not Hispanic or Latino	24 (92.3)	24 (92.3)	14 (87.5)	13 (86.7)
Unknown	1 (3.8)	1 (3.8)	1 (6.3)	1 (6.7)
Dysmorphic facial features, <i>n</i> (%)				
Lip (score 4 or 5)	15 (57.7)	14 (53.8)	10 (62.5)	6 (40.0)
Philtrum (score 4 or 5)	18 (69.2)	13 (50.0)	12 (75.0)	6 (40.0)
Palpebral fissure (≤10th percentile)	17 (65.4)	19 (73.1)	11 (68.8)	11 (73.3)
≥2 Facial features present	15 (57.7)	15 (57.7)	10 (62.5)	6 (40.0)
Growth deficiency (≤10th percentile), <i>n</i> (%)				
Height	6 (23.0)	7 (26.9)	4 (25.0)	3 (20.0)
Weight	4 (15.4)	7 (26.9)	2 (12.5)	3 (20.0)
Deficient brain growth (≤10th percentile), <i>n</i> (%)				
Occipital-frontal circumference	10 (38.5)	6 (23.1)	5 (31.3)	5 (33.3)
Alcohol exposure, <i>n</i> (%)				
Alcohol exposure confirmed	24 (92.3)	21 (80.8)	15 (93.7)	13 (86.7)
Alcohol exposure suspected	1 (3.8)	3 (11.5)	1 (6.3)	1 (6.3)
Unknown	1 (3.8)	2 (7.7)	0 (0)	1 (6.3)
Drug exposure, <i>n</i> (%)				
Drug exposure confirmed	16 (61.5)	12 (46.2)	9 (56.3)	8 (53.3)
Drug exposure suspected	4 (15.4)	5 (19.2)	2 (12.5)	1 (6.3)
Unknown	6 (23.0)	9 (34.6)	5 (31.3)	5 (33.3)
IOM diagnostic category, <i>n</i> (%)				
Fetal alcohol syndrome	5 (19.2)	4 (15.4)	3 (18.8)	1 (6.7)
Partial fetal alcohol syndrome	9 (34.6)	11 (42.3)	6 (37.5)	6 (40.0)
Alcohol-related neurodevelopmental disorder	12 (46.2)	11 (42.3)	7 (43.8)	9 (60.0)
Baseline Mullen early learning composite	84.6 ± 20.8	84.0 ± 10.9	79.8 ± 21.6	82.9 ± 11.0

¹ Values are *n* (%) or means ± SDs. No values differed significantly in the comparison between age-matched choline- and placebo-treated participants, using chi-square analysis. IOM, Institute of Medicine.

² Race and ethnicity are self-identified.

not differ at baseline and averaged 78.7% ± 8.8% of the age-adjusted Adequate Intake (16). Baseline serum values of choline, betaine, and phosphatidylcholine also did not differ (16, 17). PCA of genotype associations against the study population's characteristics also identified no significant associations with the participants' self-identified race (Figure 2A), sex (Figure 2B), treatment (Figure 2C), diagnostic category (Figure 2D), and age (Figure 2E). In all comparisons, PC1 explained only 13.7% of the association and PC2 explained just 7.2%. We then tested for genetic–phenotypic associations, first evaluating the simplest model in which the polymorphisms acted in an additive manner (AA vs. AB vs. BB). Stratification by intervention (choline vs. placebo) identified genotypes that were significantly associated with cognitive outcome in the choline-treated participants

(Table 3). Specifically, the gene variants for 14 SNPs within the choline transporter gene *SLC44A1* (listed in Table 3) were significantly associated ($P < 0.006$ for 13 SNPs; $P = 0.04969$ for 1 SNP) with the change-score (pre-/postintervention) on an elicited imitation sequential memory task, in which participants were observed to reproduce ordered pairs under immediate recall (delta_pairs_imm). These same 14 gene variants, plus 2 additional variants within *SLC44A1*, were also associated ($P < 0.005$ for 15 SNPs, $P = 0.023$ for 1 SNP) with change scores for adjacent pairs of items from the sequence (delta_adjpairs_imm). Participants having the variant in these SNPs, and also receiving the choline intervention, were more likely to show improvement in the memory task between pre- and postintervention, whereas no such associations were found

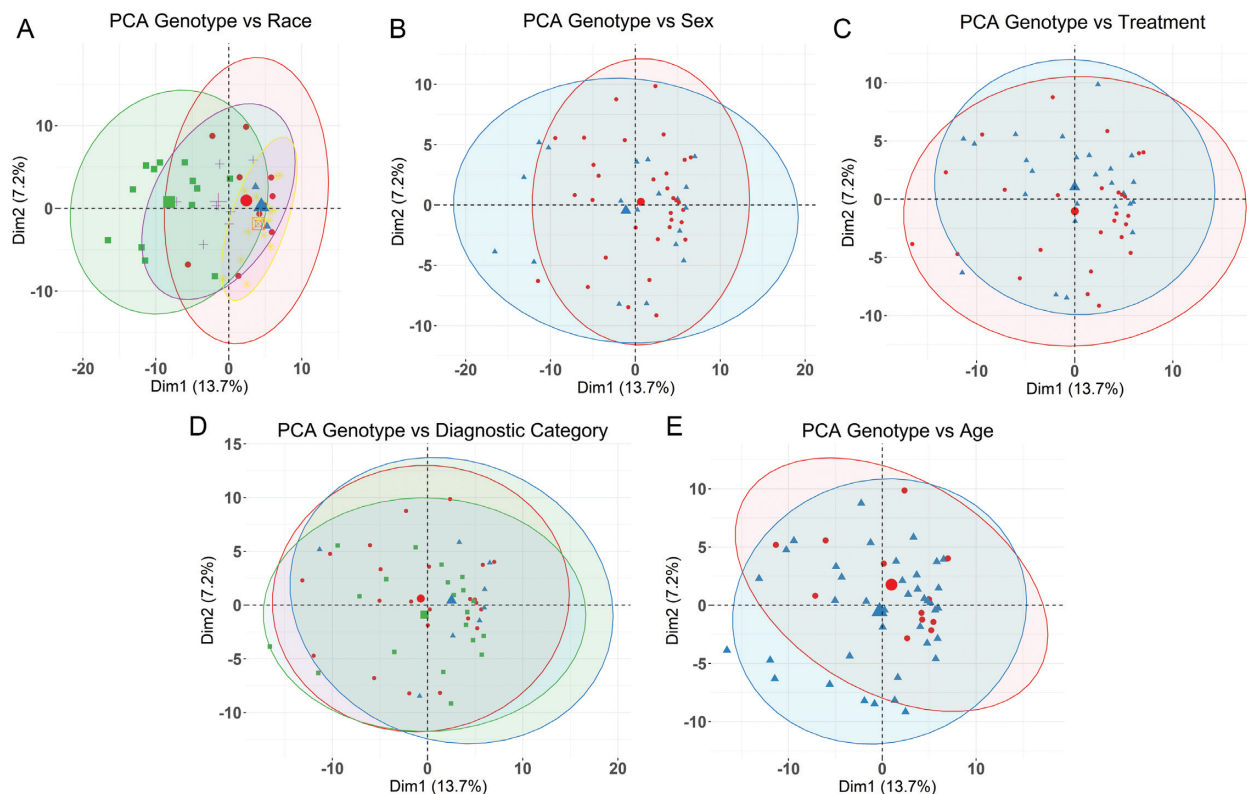


FIGURE 2 PCA of genotypes and population characteristics. PCA (FactoMineR, v2.3) was used to examine the genomic separation of all 52 participants based on race, sex, treatment, diagnostic category, or age. No significant associations between genotype and any of these population characteristics were observed in the PCA. (A) Self-identified race vs. genotype. Red circles, Native American ($n = 10$); blue triangles, Asian ($n = 2$); green squares, Black or African-American ($n = 13$); purple crosses, multiracial ($n = 7$); yellow crossed squares, White or Caucasian ($n = 19$); orange crossed squares, unknown ($n = 1$). (B) Sex vs. genotype. Red circles, female ($n = 33$); blue triangles, male ($n = 19$). (C) Treatment vs. genotype. Red circles, choline-treated ($n = 26$); blue triangles, placebo-treated ($n = 26$). (D) Diagnostic category vs. genotype. Red circles, alcohol-related neurodevelopmental disorder ($n = 23$); blue triangles, FAS ($n = 9$); green squares, partial FAS ($n = 20$). (E) Age vs. genotype. Red circles, 3 y or younger ($n = 12$); blue triangles, older than 3 y ($n = 40$). The largest symbol for each population characteristic represents the group mean for that characteristic, and the ellipse is the 95% CI surrounding that group mean. Dim1, dimension1; Dim2, dimension 2; FAS, fetal alcohol syndrome; PCA, principal components analysis.

for those participants in the placebo trial arm. The association between these effect alleles and cognitive performance was also seen in the direct comparison of individual performance in these measures, and choline participants carrying the effect alleles were more likely to show pre-/postintervention improvement compared with those carriers who did not receive choline, or those participants who lacked the effect alleles regardless of intervention (**Figure 3**). Repeating the association analysis against an assumption that these polymorphisms instead operated under a dominant (AA + AB vs. BB) or recessive (AA vs. AB + BB) genetic model found weaker, largely nonsignificant, associations. The dominant model yielded 8 SNPs within *SLC44A1* that were associated with performance in the Elicited Imitation task (delta_adjpairs_imm); these were a subset of those identified in the additive model, and their significance was an order-of-magnitude weaker ($P = 0.044$; **Supplemental Table 2**). The recessive model yielded 5 SNPs within *SLC44A1* ($P = 0.044$), all of which were shared with the dominant model. These data indicated that an additive effect of *SLC44A1* genotype best modeled the association.

Of the 32 SNPs within *SLC44A1* that were present on the array, 9 of these were in linkage disequilibrium with the 16 significant SNPs. Positional maps revealed that all 26 were distributed across

the entire gene structure for *SLC44A1*, from intron 1 through exon 16 (**Table 4**). Of particular note, rs3199966 is a coding sequence variant within exon 15, and the variant (T→G) converts serine 446 to alanine (S446A). rs2771040 (A→G) is located in the 3' untranslated region (3'-UTR) of exon 16, which is unique to the *SLC44A1* plasma membrane isoform (34). The remaining SNPs were intronic single nucleotide variants, and none were located within splice junctions or candidate microRNAs. PCA of these 26 SNPs found a significant association with self-identified race (PC1 = 56.6%; **Supplemental Figure 1**), reflecting that the effect variants are more frequent in populations with African ancestry. There was no association with sex, diagnostic category, or age.

We identified additional associations between the 243 SNP genotypes and the behavioral outcomes and physical characteristics of the choline-supplemented FASD cohort (**Table 5**). With respect to behavior, improved performance on the working memory measure of the SB-5 (stanfbi_wm) at 4-y follow-up was associated with variants within *FMO3* (rs1920149, G, $P = 0.0113$; rs909531, T, $P = 0.00512$). Variants in *MTHFD1* were associated with improved performance in the Elicited Imitation immediate memory task at baseline (rs3783731, C, $P = 0.01122$; rs8011839, C, $P = 0.01122$) and in the NIH Toolbox Dimensional Card Sort Test at the 4-y follow-up

TABLE 3 SNPs and effect alleles within *SLC44A1* associated with improved cognitive outcome in choline-treated participants¹

SNP	delta_pairs_imm ²		delta_adjpairs_imm ²			Number w/effect allele ⁵
	P	Reg. coeff. ³	P	Reg. coeff.	Effect allele ⁴	
rs10123494	0.005672	-1.0505	0.000506	-1.1232	C (T > C)	10/22
rs10820801	0.005141	-1.0476	0.001469	-1.0872	G (A > G)	9/22
rs10991639	0.005141	-1.0476	0.001469	-1.0872	A (G > A)	9/22
rs2417615	0.005141	-1.0476	0.001469	-1.0872	A (G > A)	9/22
rs2771040	0.005141	-1.0476	0.001469	-1.0872	G (A > G)	9/22
rs3199966	0.049690	-1.0516	0.001285	-1.1981	G (T > G)	9/22
rs34750132	0.005672	-1.0505	0.000506	-1.1232	G (A > G)	10/22
rs35603631	0.005672	-1.0505	0.000506	-1.1232	C (T > C)	10/22
rs4549843	0.005141	-1.0476	0.001469	-1.0872	A (G > A)	9/22
rs6479311	0.005141	-1.0476	0.001469	-1.0872	A (C > A)	9/22
rs6479313	0.005672	-1.0505	0.000506	-1.1232	G (C > G)	10/22
rs7024985	0.003171	-1.0476	0.001469	-1.0872	T (C > T)	9/22
rs7029443	0.005672	-1.0505	0.000506	-1.1232	A (T > A)	10/22
rs7865985	0.005672	-1.0505	0.000506	-1.1232	C (T > C)	10/22
rs12339823	NS	NS	0.004567	-1.1659	G (A > G)	7/22
rs16924529	NS	NS	0.023036	-1.3298	A (G > A)	5/22

¹Reg. coeff., regression coefficient; SNP, single nucleotide polymorphism.

²Associations between SNPs and outcomes were tested under an additive genetic model using the SNPAssoc package in R (v.1.9–2). *P* values were adjusted for multiple testing using Bonferroni correction. Delta_pairs_imm is the change in Elicited Imitation immediate memory task performance (pairs) between baseline (0 mo) and 9 mo; delta_adjpairs_imm is the change in Elicited Imitation immediate memory task performance (adjacent pairs of items) between baseline (0 mo) and 9 mo.

³Regression coefficient, extracted from additive model in SNPAssoc package in R (v. 1.9–2).

⁴Measured on top strand 5' → 3'. Effect allele; major > minor allele within dbSNP.

⁵Number of genotyped individuals carrying at least 1 copy of the effect allele within the choline cohort; 4 of the 26 children were missing this behavioral assessment and were excluded from this analysis.

(rs8003379, A, *P* = 0.00595). FMO3 oxidizes the microbial choline metabolite trimethylamine to trimethylamine oxide, and MTHFD1 interconverts the tetrahydrofolate one-carbon unit for purine, pyrimidine, and methionine synthesis. The unassigned SNP rs5899654 is an insertion/deletion (TAA) wherein deletion was associated with improved change-score measures from baseline to 9 mo for the Immediate Memory task in the elicited imitation (delta_pairs_imm, *P* = 0.00514; delta_adjpairs_imm, *P* = 0.00147); this SNP was merged with rs3030595 and is located within intron 1 of *SLC44A1*.

Additional, novel associations were found for physical characteristics of those receiving choline (Table 5). An allelic variant in *BHMT*, rs567754 (C→T), was weakly associated with greater height gain (*P* = 0.051) and height percentile at 9 mo (*P* = 0.047), as was a variant in the unassigned SNP (rs1275103, G; *P* = 0.0193 and *P* = 0.022, respectively) located on chromosome 5. A variant in *FMO3* (rs2064074, A, *P* = 0.0495) weakly correlated with greater weight gain. At baseline, there were no treatment-group differences in serum concentrations for choline, phosphatidylcholine, and betaine (17), and we observed no associations with these apart from a weak, positive association between baseline serum betaine and a variant in *FMO3* (rs1736560, G, *P* = 0.048). There were no genotype interactions with serum choline and its metabolites during the study intervention period.

Finally, several additional associations emerged in 2 genes involved in lipid metabolism (Table 5). A variant in the adiponectin receptor 1 (*ADIPOR1*; rs7539542, G, *P* = 0.0098) positively correlated with improved performance on the picture sequence memory task in the NIH Toolbox (nihpsmt_agecorrstandsc). For

fatty acid desaturase-2 (*FADS2*), the variant rs17156442 (C→T, *P* = 0.0023) was associated with improved performance on the immediate recall adjacent pairs task at the 4-y follow-up, and the variant rs2072114 correlated with greater height (*P* = 0.044). To explore this latter finding further, analysis across the entire set of study participants revealed novel associations between physical characteristics (height, weight) and polymorphisms in *BHMT* (*P* < 0.012), *FADS2* (*P* = 0.0306), and *SLC44A1* (*P* < 0.034; **Supplemental Table 3**). Similar novel height and weight associations were present in the placebo controls for *FADS2* (*P* < 0.025), *MDR3* (*P* < 0.046), and *SLC44A1* (*P* = 0.0074; Supplemental Table 3), and the *MTHFR* variant rs17421511 G (*P* ≤ 0.040) was associated with improved cognitive measures on the SB-5 assessment.

Discussion

Data herein may help explain the variable impact of choline supplementation upon cognitive functioning in children diagnosed with FASD. Ongoing clinical trials report lesser cognitive improvements (17, 18, 20, 21, 35) compared with the large effect sizes obtained in animal models of FASD (15). We find that, for this cohort, the benefits are masked when cognitive performance was pooled across genotypes. Stratification by genotype in *SLC44A1*, the gene coding for low-affinity choline transport across the plasma and mitochondrial membranes, revealed that choline supplementation was associated with increased sequential memory task scores for those participants who carried the minor allelic variants. Importantly, this same cognitive measure, the elicited imitation immediate performance,

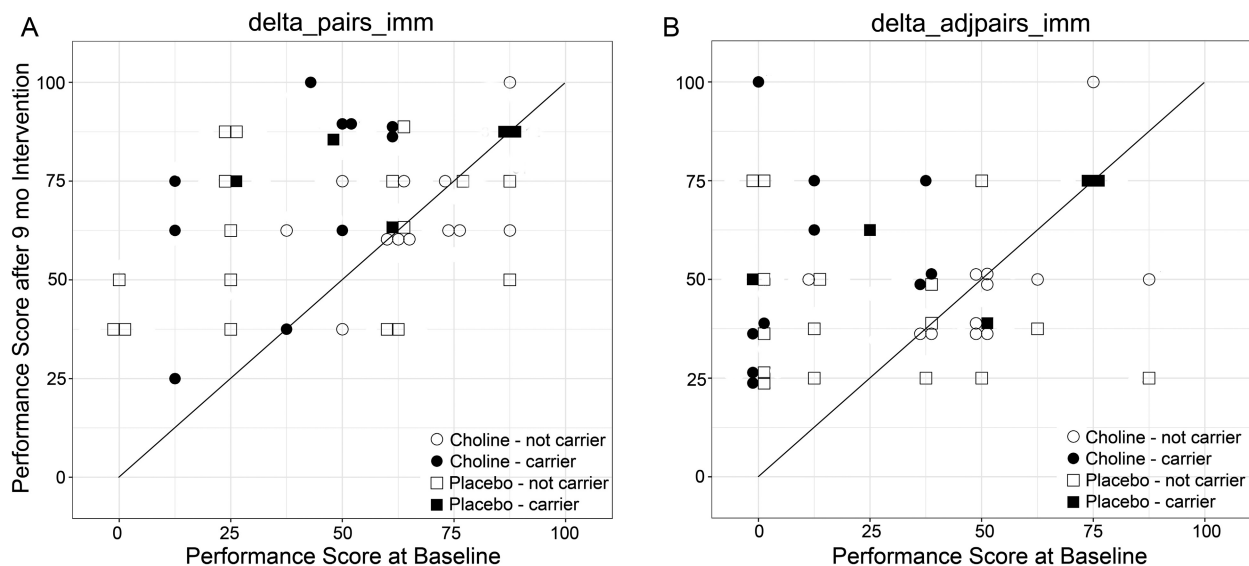


FIGURE 3 Change in cognitive performance for participants, stratified by intervention and effect allele status. Comparison of individual cognitive performance in the *delta_pairs_imm* (A) and *delta_adjpairs_imm* (B) outcomes at baseline (0 mo) and postintervention (9 mo), stratified by whether the participant carried the effect alleles (solid symbols) and received the choline (●) or placebo (■) intervention, or did not carry the effect alleles (open symbols) and received either the choline (○) or placebo (□) intervention. *delta_pairs_imm* is the change in Elicited Imitation immediate memory task performance (for pairs of items regardless of sequence order) between baseline (0 mo) and 9 mo, and *delta_adjpairs_imm* is the change in Elicited Imitation immediate memory task performance (for pairs of adjacent items placed in the correct sequence order) between baseline (0 mo) and 9 mo; for both, higher scores indicate greater positive change (improvement). Carriers receiving choline (solid circles) all reside at or above the regression line that indicates no change in performance ($R = 1.00$). Because most of the effect alleles were in linkage disequilibrium, all carriers held at least 6 effect alleles. Presented are the 44 of 52 participants who completed this task at baseline and study conclusion; sample sizes are choline carrier, $n = 10$ of 11 participants; choline not carrier, $n = 12$ of 15 participants; placebo carrier, $n = 5$ of 5 participants; placebo not carrier, $n = 17$ of 21 participants.

showed a nonsignificant trend for improvement in the original, nongenotypic analysis (17); our analysis shows that those carrying the effect variants (Table 2) in *SLC44A1* account for this trend. There is a significant a priori rationale for such a genotype interaction, as polymorphisms that modulate individual choline need have been described for multiple choline-related genes, including *SLC44A1*, *PEMT*, *CHDH*, and *BHMT*, among others (23–31). Such a gene–nutrient interaction has not been previously identified for FASD. A prior choline intervention trial in heavy-drinking pregnant women found no interactions between outcomes and the rs12325817 polymorphism in *PEMT* (35); however, potential interactions may have been difficult to identify due to the participants’ low protocol adherence. It will be important to replicate these preliminary findings in a prospective a priori trial of choline versus genotype, complemented by confirmatory post hoc analysis of other FASD–choline intervention trials (20, 21, 35), and within the general population of those diagnosed with FASD (6, 7, 9, 36).

The association between *SLC44A1* and cognitive improvement under choline supplementation is consistent with this protein’s known function. *SLC44A1*, also known as the choline transporter-like protein 1 (CTL-1), is ubiquitous and mediates the bulk of choline transport for most tissues, including brain, where it is expressed by neurons, oligodendrocytes, and endothelial cells (37, 38). *SLC44A1* is essential for brain development, and in humans its functional loss is associated with cognitive decline, ataxia, tremor, white matter deficits, hearing loss, and cerebral and cerebellar atrophy (38–40). These deficits may reflect, in part, its role in myelin compaction through interactions

with nectin-like 4, whereas its loss-of-function impairs neurite myelination (41). The predicted protein structure of *SLC44A1* features 7 transmembrane domains, and alternate splicing at its carboxy-terminus directs the protein to either the cell membrane (*SLC44A1a*; exon 16) or the mitochondria [*SLC44A1b*; exon 17; (38)].

Although most of the 25 SNPs implicating *SLC44A1* in FASD cognitive outcomes were intronic, 2 may have functional relevance. rs3199966 (T > G) is a structural variant (S644A) in the carboxy-terminus of both cell membrane and mitochondrial isoforms, and is predicted to reside in an extracellular domain (34). The rs3199966 GG genotype is associated with greater vulnerability to skeletal muscle damage (P -adjusted = 0.0493) under a low-choline diet (26). Under high choline intake (930 mg/d), women carrying the rs3199966 variants TG or GG convert more deuterated choline into betaine and methionine synthesis, an effect not observed under adequate choline intake (430 mg) (34). rs2771040 (A > G) is 4.4 kb downstream of rs3199966 and is located in the 3′-UTR of the *SLC44A1a* plasma membrane isoform, where it could potentially impact, for example, translation efficiency or structural motifs that modulate transcript stability. rs2771040 also positively correlates with more severe skeletal muscle damage (P -adjusted = 0.0024) when people are fed low-choline diets (26). Thus, both SNPs are associated with increased vulnerability to choline insufficiency. Baseline dietary choline intake in these choline-treated participants averaged 68.6% of their age-adjusted Adequate Intake (16); thus, it is possible that some individuals carrying these SNPs had an inadequate choline intake.

TABLE 4 SNPs and effect alleles within *SLC44A1* showing an association with FASD or are in linkage-disequilibrium with these SNPs¹

SNP	Nucleotide	Location	Effect allele (major > minor allele in dbSNP) ²
rs327959	105,246,149	Intron 1	Increased with T (C > T)
rs327956	105,246,635	Intron 1	Increased with A (G > A)
rs443094	105,254,404	Intron 1	Increased with C (G > C)
rs328006	105,277,527	Intron 1	Increased with C (G > C)
rs327947	105,280,309	Intron 1	Increased with G (A > G)
rs193008	105,280,525	Intron 1	Increased with C (T > C)
rs12335779	105,288,137	Intron 1	Increased with G (C > G)
rs10991611	105,301,414	Intron 2	Increased with T (C > T)
rs9644967	105,305,895	Intron 2	Increased with A (G > A)
rs10991618	105,310,238	Intron 3	Increased with A (G > A)
rs2417615	105,325,570	Intron 3	Increased with A (G > A)
rs6479311	105,347,335	Intron 4	Increased with A (C > A)
rs10991639	105,355,290	Intron 5	Increased with A (G > A)
rs7024985	105,358,537	Intron 7	Increased with T (C > T)
rs7865985	105,358,537	Intron 7	Increased with C (T > C)
rs10123494	105,359,416	Intron 7	Increased with C (T > C)
rs34750132	105,360,429	Intron 7	Increased with G (A > G)
rs6479313	105,364,024	Intron 9	Increased with G (C > G)
rs7029443	105,367,465	Intron 12	Increased with A (T > A)
rs10820801	105,367,540	Intron 12	Increased with G (A > G)
rs4549843	105,368,325	Intron 12	Increased with A (G > A)
rs35603631	105,380,371	Intron 13	Increased with C (T > C)
rs16924529	105,382,872	Intron 13	Increased with A (G > A)
rs3199966	105,385,482	Exon 15	Increased with G (T > G)
rs12339823	105,387,394	Intron E15–E16	Increased with G (A > G)
rs2771040	105,389,918	Exon 16 3'-UTR	Increased with G (A > G)

¹E, exon; SNP, single nucleotide polymorphism; 3'-UTR, 3'-untranslated region.

²Measured on top strand 5' → 3'. All of these alleles are single nucleotide variants.

Biochemical studies of *SLC44A1* offer additional insight into how it could modulate differential sensitivity to choline status. Choline intake modulates *SLC44A1* activity (42–44), and under low choline *SLC44A1* can be internalized via phosphorylation at serine 12 and serine 13 (43). A reduction in *SLC44A1* membrane abundance has been hypothesized to favor choline uptake by the high-affinity transporter *SLC5A7*, which is enriched on cholinergic neurons and with the outcome of prioritizing acetylcholine synthesis when choline is limiting (37). This concentration-dependent activity of *SLC44A1* may explain why cognitive enhancement was only associated with those carrying the effect variants in *SLC44A1* (Table 2). As shown by da Costa et al. (26), those carrying these variants have greater vulnerability for poor choline status under conditions of low choline intake and would therefore derive greater benefit from choline supplementation. Functional studies are necessary to define the underlying mechanism; for example, the effect variant might be associated with reduced transporter activity, or with a greater reduction in membrane abundance when choline intake is low, and thus causing a greater reduction in cellular choline content. Alternately, the effect allele might confer greater membrane abundance under choline supplementation, and we note that, under supraphysiological choline intake, carriers of the effect allele in rs319996 direct greater quantities of choline into methylation via PEMT, rather than into the direct synthesis of phosphatidylcholine (23); this observation may offer mechanistic insight into how choline improves cognition in this FASD

subpopulation. Conclusions regarding these putative mechanisms await functional testing of *SLC44A1* and its allelic variants.

This study has multiple limitations. A genetic association analysis was not a primary outcome of the original study, and thus our analysis was likely underpowered to detect choline–gene associations for many of the study’s measures. A significant benefit was only identified for *SLC44A1*, and the size effects were an order-of-magnitude smaller for polymorphisms in other choline-related genes known to affect choline requirements, including *FMO3*, *BHMT*, and *MTHFD*; their lower significance may be a limitation of the small cohort size. Our failure to identify parallel genetic-cognitive associations at the 4-y follow-up may similarly reflect the underpowered sample size. We did not identify associations with additional cognitive measures within the choline intervention cohort; the benefit was observed for the easiest task, and perhaps supplementation for a longer duration, or at younger ages, will confer greater benefit. Supporting this is the demonstration that alcohol-abusing women who were most compliant with a gestational choline supplement gave birth to children with the greatest choline-related cognitive performance (35). Finally, there was largely no association between these polymorphisms and choline-related metabolites, and thus plasma values did not reflect who gained the greatest benefit from the choline supplement.

In summary, we have identified genotypes affecting choline metabolism in individuals diagnosed with FASD and that are associated with significant cognitive improvement in an

TABLE 5 Additional SNP associations within choline-treated participants¹

Gene	SNP	<i>P</i> ²	Reg. coeff.	Outcome ³	Effect allele (major > minor allele in dbSNP) ⁴	Number w/effect allele ⁵
<i>ADIPOR1</i>	rs7539542	0.00981	− 1.2034	nihpsmt_agecorrstandsc	Increased with G (C > G)	9/15
<i>BHMT</i>	rs567754	0.05079	0.8758	height_z_physical.11	Increased with C (C > T)	15/23
	rs567754	0.04701	0.8791	height_pile_physical.11	Increased with C (C > T)	15/23
<i>CHDH</i>	rs881883	0.02386	− 1.1318	ibb_tscore21	Increased with C (T > C)	7/15
<i>FADS2</i>	rs2072114 ⁶	0.04435	1.4352	height_physical.11	Increased with A (A > G)	8/23
	rs17156442 ⁶	0.00231	− 2.0158	imt_adjpairs_imm_med.11	Increased with T (C > T)	4/23
<i>FMO3</i>	rs1920149	0.01134	− 1.1290	stanfbi_wm	Increased with G (A > G)	11/14
	rs909531	0.00512	1.1902	stanfbi_wm	Increased with T (T > C)	7/14
	rs2064074	0.04953	0.9732	weight_z_physical.3	Increased with A (A > G)	15/21
	rs1736560	0.04792	− 0.0892	bloodlevel_betaine.1	Increased with G (C > G)	12/22
<i>MTHFD1</i>	rs8003379	0.00595	1.6801	nihdccc_agecorrstandsc	Increased with A (A > C)	7/15
	rs8003379	0.02407	1.6144	nihdccc_fullcortsc	Increased with A (A > C)	7/15
	rs2295640	0.00648	− 2.1758	blood_pressure_diastolic.8	Increased with G (C > G)	3/21
	rs3783731	0.01122	1.1129	imt_comp_imm_med.1	Increased with C (C > T)	8/25
	rs8011839	0.01122	1.1129	imt_comp_imm_med.1	Increased with C (C > T)	8/25
<i>MDR3</i>	rs31653	0.05053	2.3077	memnames_t3total	Increased with G (G > A)	2/15
<i>PEMT</i>	rs7214988	0.04579	− 1.9685	iib_avg_int_adjpairs	Increased with G (C > G)	3/15
	rs7224725	0.05131	− 2.3063	iib_avg_int_adjpairs	Increased with T (C > T)	2/15
Unassigned	rs1275103	0.01929	− 0.9149	height_z_physical.11	Increased with G (C > G)	17/23
	rs1275103	0.02179	− 0.9102	height_pile_physical.11	Increased with G (C > G)	17/23
	rs5899654 ⁷	0.00514	− 1.0476	delta_pairs_imm	Increased with D (I > D)	9/22
	rs5899654 ⁷	0.00147	− 1.0872	delta_adjpairs_imm	Increased with D (I > D)	9/22
	rs998671	0.00509	1.6972	activity_level.11	Increased with G (G > A)	6/24

¹*ADIPOR1*, adiponectin receptor 1; D, deletion; *BHMT*, betaine-homocysteine S-methyltransferase; *CHDH*, choline dehydrogenase; *FADS2*, fatty acid desaturase-2; *FMO3*, flavin monooxygenase-3; I, insertion; *MDR3*, multidrug resistance protein 3; *MTHFD1*, methylenetetrahydrofolate dehydrogenase-1; *PEMT*, Phosphatidylethanolamine N-Methyltransferase; Reg. coeff., regression coefficient [extracted from additive model in SNPpassoc package in R (v. 1.9–2)]; SNP, single nucleotide polymorphism.

²Associations between SNPs and outcomes were tested under an additive genetic model using the SNPpassoc package in R (v.1.9–2). *P* values are adjusted for multiple testing using Bonferroni correction.

³Outcomes are defined in Table 1.

⁴Measured on top strand, 5′ → 3′. All other alleles are single nucleotide variants.

⁵Number of genotyped individuals carrying the effect allele within the choline cohort; some outcomes were missing for some children and thus were excluded from that analysis.

⁶Also observed in analysis of all participants (Supplemental Table 3); *P* = 0.03062 for rs2072114, *P* = 0.03616 for rs17156442.

⁷Merged with rs3030595 (10 May 2012); housed in intron 1 of *SLC44A1* (TAA > deletion).

early childhood intervention trial with choline. *SLC44A1* also mediates placental choline transport (38), and we predict that a similar association will likely emerge from the gestational choline intervention trials. Polymorphisms that affect the activity of additional choline-related genes will likely have similar influences, and lacked a robust association here, perhaps because the cohort was underpowered. It is challenging to prevent FASD due to the complex social issues that surround binge drinking, and we have few interventions to improve the daily function of those who are affected. Our data support the continued use of supraphysiological choline intake to improve cognitive function in those who are diagnosed with FASD, and the need to incorporate similar genetic analyses in future choline intervention trials for FASD and for other cognitive disorders.

The authors' responsibilities were as follows—JRW, SHZ, MKG, and JKE: designed the research; JRW, CJB, KES, JKE, and SHZ: conducted the research; MSV: analyzed the data; SMS, MSV, JRW, and SHZ: wrote the manuscript; SMS and JRW: have primary responsibility for final content; and all authors: read and approved the final manuscript. SHZ is the founder of SNP Therapeutics, he is on advisory boards for Ingenuity Brands

and ByHeart, and has received grant funding from Balchem; all of these companies have an interest in choline. The other authors report no conflicts of interest.

Data Availability

Deidentified data described in the manuscript, code book, and analytic code will be made available upon request.

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