Choline and Risk of Neural Tube Defects in a Folate-fortified Population

Gary M. Shaw,[a] *Richard H. Finnell,*[b] *Henk J. Blom,*[c] *Suzan L. Carmichael,*[d] *Stein Emil Vollset,*[e] *Wei Yang,*[d] *and Per M. Ueland*[f]

Background: Folic acid is known to reduce risk of neural tube defects (NTDs). Even so, NTDs continue to occur despite individual supplementation or population fortification with folic acid. We investigated other nutrients related to one-carbon metabolism that may affect NTD risk.

Methods: This prospective study included data from more than 180,000 pregnant women in California from 2003 through 2005. Midpregnancy serum specimens were linked with delivery information regarding the presence of a NTD, another structural malformation, or no malformation in the fetus. We identified 80 NTD-affected pregnancies (cases) and we randomly selected 409 pregnancy controls. Serum specimens were tested for methylmalonic acid, homocysteine, cysteine, methionine, total choline, betaine, cystathionine, vitamin B6, folate, vitamin B12, riboflavin, and creatinine.

Results: We observed elevated NTD risks associated with lower levels of total choline, and reduced risks with higher levels of choline. Specifically, we observed an odds ratio of 2.4 (95% confidence interval = 1.3–4.7) associated with the lowest decile and an odds ratio of 0.14 (0.02–1.0) associated with the highest decile, both relative to the 25th–74th percentiles of the control distribution. These data did not show meaningful differences between cases and controls for any other analytes.

Conclusions: This is the first study to investigate total choline in NTD-affected pregnancies. Our findings for choline, for which low levels were a risk factor and higher levels were a protective factor for NTDs, may offer a useful clue toward understanding the complex etiologies of NTDs in an era of folic acid fortification of the food supply.

(*Epidemiology* 2009;20: 714–719)

Neural tube defects (NTDs) are common, costly, and deadly human congenital anomalies whose etiologies remain largely unknown. The preventive effects of folic acid on NTD risk have been well established.[1–7] The epidemiologic evidence is robust, including, (1) consistent results from multiple study designs conducted in different populations,[1] (2) a decline in prevalence of NTDs since the US food supply was fortified with folic acid in 1998,[2] (3) lower red cell and serum folate levels among women who previously have had a child with a NTD,[3,4] and (4) elevated NTD risks associated with exposures to folate antagonist medications.[5]

Lowered serum levels of vitamin B12, independent of folate, have been associated with increased risks of NTDs. Ray and Blom[6] recently synthesized evidence from 17 epidemiologic studies that investigated the association between maternal vitamin B12 status and NTD risk, and concluded there was evidence for a moderate risk between B12 insufficiency and NTD risk. Other nutrients and nutrition-related factors associated with reduced NTD risks include increased intakes of methionine,[7] zinc,[8] vitamin C,[9] and choline[10] (a dietary component of lecithin).

Despite the evidence for folic acid, the underlying mechanisms by which folates contribute to reductions in NTD risks have not been elucidated. Thus, it remains unknown why NTDs continue to occur despite individual supplementation or population fortification with folic acid. In a landmark study more than 3 decades ago, Smithells and colleagues[11] observed lower levels of folate as well as vitamin C in serum of pregnant women with NTD fetuses relative to women without NTD fetuses. Numerous research efforts have since been made to investigate early pregnancy blood measures of primarily folates and vitamin B12.[3,4,6,12–16] The current prospective study extends these research efforts by investigating these measures as well as other nutrients related to one-carbon metabolism, particularly choline (a methyl carrier), using midpregnancy sera obtained from a large, folate-fortified population in California.

Submitted 30 July 2008; accepted 20 November 2008.

From the [a]Stanford University School of Medicine, Stanford, California; [b]Institute of Biosciences and Technology, The Texas A&M University System Health Science Center, Houston, TX; [c]Department of Clinical Chemistry, VU University Medical Center, Amsterdam, The Netherlands; [d]March of Dimes California Research Division, Children's Hospital Oakland Research Institute, Oakland, CA; [e]Medical Birth Registry of Norway, Norwegian Institute of Public Health, Bergen, Norvege; and [f]Section for Pharmacology, Institute of Medicine, University of Bergen and Haukeland University Hospital, Bergen, Norway.

Supported by the Centers for Disease Control and Prevention, Center of Excellence Award U50/CCU913241, by NIH/NINDS R01 NS050249, and the Foundation to promote research into functional vitamin B12-deficiency.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the California Department of Public Health.

Correspondence: Gary M. Shaw, March of Dimes California Research Division, Children's Hospital Oakland Research Institute, 5700 Martin Luther King Jr Way, Oakland, CA 94609. E-mail: gmshaw@stanford.edu.

ISSN: 1044-3983/09/2005-0714
DOI: 10.1097/EDE.0b013e3181ac9fe7

METHODS

Data Sources

We conducted a nested case-control study within a large and unique midpregnancy serum specimen bank in California. Sera were collected from women as part of the California Expanded AFP (alpha-feto-protein) program that screens pregnant women for NTDs and cytogenetic abnormalities. Specimens were collected during the 15th–18th week of pregnancy from approximately 70% of women who resided in selected regions of California (Orange, San Diego, and Central Valley counties). The collection and processing of specimens was as follows: (1) samples were taken at draw stations using BD Vacutainer 3.5 mL serum separator tubes (BD, Franklin, Lakes, NJ) with no anticoagulants or preservatives and centrifuged; (2) samples were received by designated clinical laboratories at room temperature, on average 3.0 days after draw; (3) AFP screening assays were run on samples, usually on the day received; (4) samples were refrigerated up to 7 days if further testing was necessary; (5) samples were sent on cold packs via overnight mail to the serum storage bank; and (6) samples were aliquoted, labeled with barcodes, and frozen at –70°C within an average of 3.5 days of receipt at the serum storage bank.

Each woman's serum specimen was linked with delivery outcome information to determine whether her fetus had a NTD or any other structural malformation ascertained by the California Birth Defects Monitoring Program.[17] The study included deliveries that were liveborn, stillborn (fetal deaths at greater than 20 weeks' gestation), or electively terminated based on prenatal diagnoses. Among the more than 180,000 pregnancy specimens collected for testing in the period 2003–2005, we identified 80 NTD-affected pregnancies (cases). Of the 80 cases, 31 had spina bifida and 49 had anencephaly. We also randomly selected 409 pregnancy specimens that were collected during the same time period and for which no malformation had been identified at delivery (controls). All samples were obtained with approval from the California Health and Welfare Agency Committee for the Protection of Human Subjects.

Serum specimens for the 489 cases and controls were shipped on cold packs to the University of Bergen for analyte measurements. The analytes measured were methylmalonic acid, total homocysteine, cysteine, methionine, total choline, betaine, cystathionine, pyridoxal phosphate, pyridoxal, pyridoxic acid, folate, cobalamin (vitamin B12), riboflavin, and creatinine. To assess cigarette smoking, the metabolite cotinine was measured. "No smoke exposure" was defined as values <5 nmol/L, and "any smoke exposure" was defined as ≥ 5 nmol/L. Details about all laboratory assays except total choline can be found elsewhere.[18] Total choline was measured after conversion of choline esters to free choline in the presence of phospholipase D. Serum/plasma sample of 45 μL was mixed with 18 μL solution containing phospholipase D (Sigma Chemical Company, St. Louis, MO, 2.8 U/μL), CaCl2 (86 mM) and Triton (0.44%). 30 μL dithioerythritol (147mM) and 60 μL TCA containing 400 μM d7Choline were added to the incubation mixture. After centrifugation, the supernatant was analyzed by LC-MS/MS using a method optimized for the determination of free choline.[19] All laboratory analyses were performed without awareness of case and control status.

Statistical Analysis

We used *t* tests to compare mean levels of analytes between cases and controls. We also estimated risks using odds ratios (ORs) and 95% confidence intervals (CIs) (SAS 9.1; SAS Institute, Cary, NC). Models were constructed to assess effects associated with categories of the measured analytes. Specifically, we categorized measures as $<$25th percentile, 25th–74th percentile, and $\geq$75th percentile based on the distribution of each analyte among controls. The 25th–74th percentile was used as the reference group. We analyzed data for linear (logistic regression) and nonlinear (spline regression) effects and found no evidence for the latter. Intake forms associated with the screening program provided data on maternal race/ethnicity (Hispanic; white, nonHispanic; Asian; Black; other) and maternal age ($<$25; 25–29; 30–34; and $>$34 years). These factors together with cigarette smoke exposure (defined by cotinine levels) were considered as covariates.

RESULTS

Table 1 provides background characteristics of the study population. As expected, there were more Hispanic pregnancies in the case group. Cases were over-represented in the age group 25–29 and under-represented in the group 30–34. Table 2 shows means and standard deviations of each analyte. We observed a substantially lower mean level in

TABLE 1. Characteristics of Mother's With NTD-affected Pregnancies in California 2003–2005

	NTD Cases (n = 80)	Controls (n = 409)
	No. (%[a])	No. (%[a])
Race/ethnicity		
Hispanic	53 (66)	196 (48)
White non-Hispanic	14 (18)	143 (35)
Asian	7 (9)	37 (9)
Black	1 (1)	12 (3)
Other	5 (6)	20 (5)
Age (years)		
$<$25	28 (35)	132 (32)
25–29	27 (34)	91 (22)
30–34	15 (19)	137 (34)
$>$34	10 (13)	49 (12)

[a]Percentages may not equal 100 owing to missing data or rounding.

TABLE 2. Mean Values of Selected Maternal Biochemical Measures in Mid-pregnancy Serum Specimens Between NTD-affected and Unaffected Deliveries, California 2003–2005

Serum Measurements	NTD Cases (n = 80) Mean (SD)	Controls (n = 409) Mean (SD)	Difference of 2 Means (95% CI)
Homocysteine (tHcy) (umol/L)	7.12 (3.76)	6.96 (3.75)	0.16 (−0.74 to 1.06)
Methylmalonic acid (umol/L)	0.14 (0.05)	0.15 (0.05)	−0.01 (−0.02 to 0.01)
Folate (nmol/L)	33.68 (16.55)	36.48 (20.88)	−2.80 (−7.66 to 2.07)
Vitamin B_{12} (pmol/L)	321.13 (139.33)	323.47 (165.23)	−2.34 (−41.32 to 36.64)
Pyridoxal phosphate (nmol/L)	76.66 (87.43)	72.19 (63.17)	4.47 (−11.86 to 20.81)
Pyridoxal (nmol/L)	46.72 (48.29)	63.19 (143.87)	−16.47 (−48.65 to 15.70)
Pyridoxic acid (nmol/L)	42.87 (46.40)	64.47 (153.27)	−21.60 (−55.81 to 12.60)
Riboflavin (nmol/L)	46.94 (36.52)	39.88 (29.86)	7.06 (−0.43 to 14.56)
Total choline (mmol/L)	2.77 (0.36)	2.98 (0.41)	−0.21 (−0.31 to −0.11)
Betaine (umol/L)	17.35 (4.47)	16.69 (4.64)	0.66 (−0.45 to 1.77)
Methionine (umol/L)	35.85 (10.16)	36.28 (9.72)	−0.43 (−2.78 to 1.93)
Total cysteine (tCys) (umol/L)	237.35 (48.04)	234.31 (46.34)	3.04 (−8.17 to 14.23)
Cystathionine (umol/L)	0.1137 (0.0408)	0.1154 (0.0547)	−0.002 (−0.014 to 0.011)

cases than in controls for total choline (at least one-half standard deviation).

Given that comparisons of mean analyte values may not adequately reveal differences in the tails of the distribution, we explored lower and upper quartiles (cutpoints defined by the control distribution). As shown in Table 3, the strongest associations were for total choline. Adjustment for maternal age and race/ethnicity did not change the results for total choline. Similarly, the exclusion of women with cigarette smoking exposure (3 case and 32 control women) did not produce substantially different results. Because effects were observed for both lower and higher levels of total choline, we investigated even more restricted levels (deciles rather than quartiles) to determine if effects became more pronounced. The odds ratio was 2.4 (1.3–4.7) for the lowest decile (<2.49 mmol/L; 18 cases and 40 controls), and 0.14 (0.02–1.0) for the highest decile (>3.50 mmol/L; 1 case and 40 controls), both relative to the 25th–74th percentile. The Figure shows the relationship between total choline and NTD-affected pregnancies (Generalized Additive Models-plot). The odds ratio associated with a 1-unit increase in total choline is 0.24 (95% CI = 0.12–0.48).

We also explored the phenotypes anencephaly and spina bifida separately for effects associated with lower and upper quartiles of total choline. Results were similar in magnitude to those for all NTDs combined, but precision was compromised owing to restricted sample size. For anencephaly, the odds ratios for lower and upper quartiles (relative to the 25th–74th percentile) were 1.8 (0.96–3.4) and 0.34 (0.11–1.0). For spina bifida, the odds ratios were 1.8 (0.80–4.0) and 0.60 (0.19–1.8).

DISCUSSION

We used prospectively collected samples to examine potential associations between several serum nutrients related to one-carbon metabolism and NTD risk. We found no difference in midpregnancy serum folate levels between pregnancies with NTDs and those with no structural malformation. This absence of an association with serum folate might be expected given that women in this study were from a population whose food supply was fortified with folic acid. It is also likely that most women took prenatal supplements containing folic acid as well as other nutrients at the time of serum sampling. However, we have no information on dietary and supplement intake.

We found a strong linear association of total choline with decreased NTD risk. Choline, known primarily in the diet as a component of lecithin, is key to several metabolic processes. Like folate, choline is involved in one-carbon metabolism, it is used for the synthesis of cell membrane phospholipids, and it is a precursor of the neurotransmitter, acetylcholine.[20–25] The demand for choline is thought to be higher during pregnancy.[24] Our observed association with choline is unlikely to be explained by differential use of prenatal vitamin supplements between case and control mothers because choline is not a typical component of multivitamin supplements.

The findings with choline are supported by previous epidemiologic data indicating that choline intake may be associated with NTD risk.[10] Our findings are also supported by known biologic underpinnings. Choline, folate, and methionine are highly interrelated in one-carbon metabolism, and an alteration in one affects the others.[22] Thus, choline deficiency could affect folate and homocysteine metabolism.[25] Methylation of DNA can be influenced by dietary contributions of methyl donors such as choline, folate, and methionine. A less than optimal methyl-donor supply and DNA methylation has been a suggested area for research efforts for certain human birth defects[26] and disruption of

TABLE 3. Effect Estimates for NTD-affected Pregnancies Associated With Selected Maternal Biochemical Measures in Mid-pregnancy Serum Specimens

Percentile Measure[a]	No. Cases (n = 80)	No. Controls (n = 409)	Odds Ratio (95% CI)	Adjusted Odds Ratio[b] (95% CI)
Total homocysteine				
<4.90	15	101	0.7 (0.4–1.3)	0.6 (0.3–1.2)
4.90–7.14[c]	43	205	1.0	1.0
≥7.15	22	103	1.0 (0.6–1.8)	1.1 (0.6–1.9)
P for trend			0.34	0.17
Methylmalonic acid				
<0.12	29	98	1.7 (1.0–2.9)	1.5 (0.8–2.6)
0.12–0.16[c]	36	206	1.0	1.0
≥0.17	15	105	0.8 (0.4–1.6)	1.0 (0.5–1.9)
P for trend			0.03	0.18
Folate serum				
<23.32	21	102	1.0 (0.6–1.8)	0.9 (0.5–1.6)
23.32–43.60[c]	42	204	1.0	1.0
≥43.61	17	103	0.8 (0.4–1.5)	0.9 (0.5–1.7)
P for trend			0.54	0.95
Vitamin B_{12}				
<223.80	18	101	1.0 (0.5–1.8)	1.0 (0.5–1.8)
223.80–381.39[c]	37	203	1.0	1.0
≥381.40	24	102	1.3 (0.7–2.3)	1.2 (0.7–2.2)
P for trend			0.40	0.49
Pyridoxal phosphate				
<35.25	17	102	0.8 (0.4–1.5)	0.8 (0.4–1.5)
35.25–79.88[c]	41	204	1.0	1.0
≥79.89	21	103	1.0 (0.6–1.8)	1.1(0.6–2.0)
P for trend			0.58	0.39
Pyridoxal				
<18.51	19	102	1.0 (0.5–1.8)	1.0 (0.6–1.9)
18.51–51.96[c]	39	204	1.0	1.0
≥51.97	21	103	1.1 (0.6–1.9)	1.2 (0.7–2.3)
P for trend			0.79	0.64
Pyridoxic acid				
<15.70	23	102	1.0 (0.6–1.8)	0.9 (0.5–1.6)
15.70–62.40[c]	44	204	1.0	1.0
≥62.41	12	103	0.5 (0.3–1.1)	0.6 (0.3–1.2)
P for trend			0.10	0.36
Riboflavin				
<22.34	18	102	1.1 (0.6–2.0)	1.0 (0.5–2.0)
22.34–46.65[c]	33	204	1.0	1.0
≥46.66	28	103	1.7 (1.0–2.9)	1.5 (0.8–2.7)
P for trend			0.16	0.23
Total choline				
<2.71	33	100	1.8 (1.1–3.0)	1.8 (1.0–3.0)
2.71–3.20[c]	38	206	1.0	1.0
≥3.21	8	102	0.4 (0.2–0.9)	0.4 (0.2–1.0)
P for trend			0.0003	0.0006
Betaine				
<13.55	16	99	0.8 (0.4–1.6)	0.8 (0.4–1.6)
13.55–18.79[c]	40	206	1.0	1.0
≥18.80	24	104	1.2 (0.7–2.1)	1.2 (0.7–2.2)
P for trend			0.31	0.24
Methionine				
<29.41	23	102	1.3 (0.7–2.3)	1.4 (0.8–2.5)
29.41–41.32[c]	36	204	1.0	1.0
≥41.33	21	103	1.2 (0.6–2.1)	1.2 (0.7–2.2)
P for trend			0.75	0.71
Total cysteine				
<207.50	20	102	1.1 (0.6–2.0)	1.0 (0.5–1.8)
207.50–245.59[c]	37	204	1.0	1.0
≥245.60	23	103	1.2 (0.7–2.2)	1.2 (0.7–2.2)
P for trend			0.69	0.49
Cystathionine				
<0.08	19	85	1.3 (0.7–2.3)	1.2 (0.6–2.2)
0.08–0.12[c]	37	209	1.0	1.0
≥0.13	24	115	1.2 (0.7–2.1)	1.1 (0.6–2.0)
P for trend			0.90	0.91

[a]Categories were constructed corresponding to <25, 25–74, and >75 percentiles, based on distributions among control mothers.
[b]Odds ratio adjusted for maternal race/ethnicity and age.
[c]Reference category.

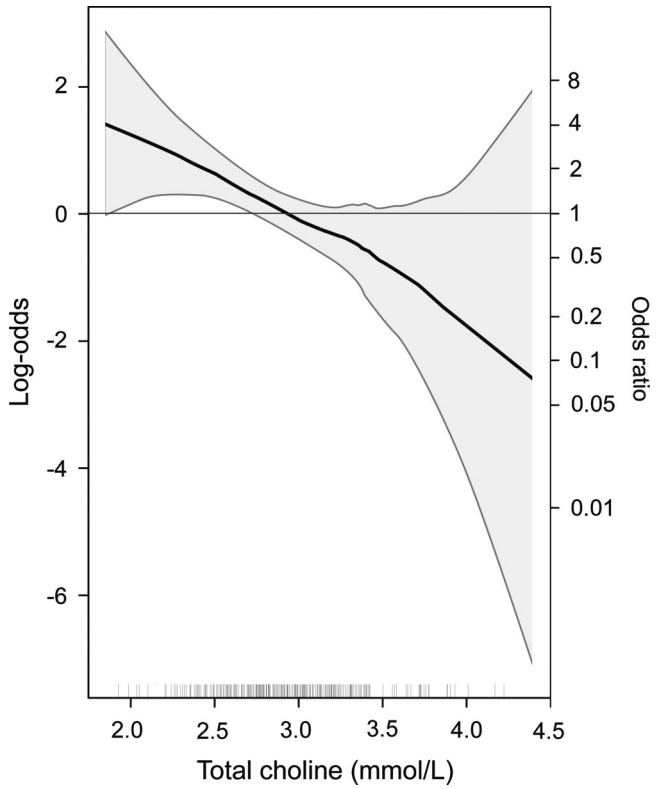

FIGURE. Log-odds of NTD-affected pregnancies associated with total choline measured in midpregnancy serum specimens. Predicted log-odds estimated from the Generalized Additive logistic regression Model (GAM). The log-odds scale is centered (ie, set to zero at the mean total choline level).

embryonic methylation has been demonstrated in experimental systems to be linked to NTDs.[27] However, findings from one experimental study of cultured mouse embryos casts some doubt on whether choline or betaine serve as methyl donors during neurulation, in that betaine homocysteine methyltransferase was not expressed until neurulation was almost complete.[28]

Alternatively, choline may affect growth in early embryogenesis because it is a precursor to phosphatidylcholine, a major component of cell membranes. It has been observed that inhibiting choline uptake and metabolism in mouse embryos results in NTDs[29] and that knockouts in genes important for mediating choline to phosphatidylcholine conversion result in early embryonal lethality.[30,31] A plausible mechanism could be the following: phosphatidylcholine is needed for cell membrane assembly; cell membrane assembly is in critical demand in a developing fetus; nearly all choline uptake by the embryo is converted to phosphatidylcholine; and embryos at the time of neurulation cannot de novo synthesize phosphatidylcholine because the enzyme phosphatidylethanolamine N-methyl transferase is not active at this stage of development.[28] Thus, the latter component places the developing embryo reliant on uptake of choline from the mother.

Another possible mechanistic role mediated by choline for improper neural tube closure may be via apoptosis.[25] Regulation of apoptosis is important to the development of the neural tube.[32] As an alternative to these proposed mechanisms for choline, it has been observed in mouse model systems that degeneration of exposed embryonic neural tissue releases both neuronal and glial proteins into amniotic fluid and that these proteins increase as gestation proceeds.[33] Thus, it is possible that a degenerating neural tube requires increased membrane synthesis owing to a repair response with a consequent reduction in maternal circulating choline levels. We are unable, however, to address this theoretical possibility further in our data.

We know of no previous studies that have directly assessed choline levels in NTD-affected pregnancies. Previous studies have investigated other analytes investigated here, including homocysteine,[34–36] folate,[3,4,12–16,36] methionine,[34] methylmalonic acid,[37] vitamin B12,[6,12–16,38] and vitamin B6.[34] In general, studies have observed elevated NTD risks associated with elevated levels of homocysteine, lowered levels of serum folate, and lowered levels of vitamin B12. Direct comparisons between our results and earlier research is complicated by several factors, including: (1) design differences such as sample collection during pregnancy versus postdelivery, sometimes years postdelivery; (2) variations in vitamin supplement content and use by the studied populations; (3) underlying dietary folic acid fortification differences in study populations; and (4) differences in specificity and sensitivity of analytes measured. For example, Ray and colleagues[38] recently observed that low maternal holotranscobalamin was associated with increased risks of NTDs.

Even though the samples in our study were collected during pregnancy, they were nonetheless collected on average 12 weeks after closure of the neural tube. If the resulting error in measurement biases our results, it is likely to result in an underestimate of measured effects. A second limitation is potential degradation of analytes between collection and analysis. Folate may degrade when frozen at higher temperatures (–20°C) than were used for samples in this study (–80°C).[39] Such degradation would likely be nondifferential to case and control status and therefore tend to underestimate real effects. Moreover, the average length of time between collection and frozen storage was similar between cases and controls. We explored whether even small differences between cases and controls influenced observed effects with total choline. Analyses that incorporated length of time into models produced even stronger odds ratios. We conducted experiments to explore the stability of total choline at room temperature and found that this analyte was stable for at least 8 days at room temperature. Specimens analyzed in the current study were, on average, at room temperature for only 3 days (range 2–4 days). Other limitations include a lack of information on supplemental and dietary intake of nutrients, a relatively small sample size that reduced power in some comparisons, and the inability to investigate allelic variants of genes involved in the biosynthesis of these nutrients, eg, folate, choline, and B12. Additional study of genetic variants coupled with analyte measures could be informative owing to the known or suspected function of selected genes.

For more than 3 decades, evidence has accumulated to show that periconceptional nutrient intakes—particularly folate—lower risks of NTD-affected pregnancies. Although fortification of the US food supply with folic acid is associated with a decreased prevalence of NTDs,[2] there is still a substantial population burden of these serious birth defects. Interestingly, Benevenga[40] has recently posited that betaine, a metabolite of choline, should be considered along with folate as a dietary supplement to reduce NTD risk. Our results showing an association with serum levels of choline (the precursor of betaine) offers an additional clue toward understanding the complex etiologies of NTDs in the era of folic acid fortification of the food supply. This result needs to be replicated in other settings, potentially through a designed trial, before firmer inferences can be drawn or recommendations made about choline.

REFERENCES

1. MRC-Vitamin-Study-Research-Group. Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. *Lancet*. 1991;338:131–137.
2. Williams LJ, Mai CT, Edmonds LD, et al. Prevalence of spina bifida and anencephaly during the transition to mandatory folic acid fortification in the United States. *Teratology*. 2002;66:33–39.
3. Yates JR, Ferguson-Smith MA, Shenkin A, Guzman-Rodriguez R,

White M, Clark BJ. Is disordered folate metabolism the basis for the genetic predisposition to neural tube defects? *Clin Genet*. 1987;31:279–287.
4. Kirke PN, Molloy AM, Daly LE, Burke H, Weir DG, Scott JM. Maternal plasma folate and vitamin B12 are independent risk factors for neural tube defects. *Q J Med*. 1993;86:703–708.
5. Hernandez-Diaz S, Werler MM, Walker AM, Mitchell AA. Folic acid antagonists during pregnancy and the risk of birth defects. *N Engl J Med*. 2000;343:1608–1614.
6. Ray JG, Blom HJ. Vitamin B12 insufficiency and the risk of fetal neural tube defects. *QJM*. 2003;96:289–295.
7. Shaw GM, Velie EM, Schaffer DM. Is dietary intake of methionine associated with a reduction in risk for neural tube defect-affected pregnancies? *Teratology*. 1997;56:295–299.
8. Velie EM, Block G, Shaw GM, Samuels SJ, Schaffer DM, Kulldorff M. Maternal supplemental and dietary zinc intake and the occurrence of neural tube defects in California. *Am J Epidemiol*. 1999;150:605–616.
9. Schorah CJ, Wild J, Hartley R, Sheppard S, Smithells RW. The effect of periconceptional supplementation on blood vitamin concentrations in women at recurrence risk for neural tube defect. *Br J Nutr*. 1983;49:203–211.
10. Shaw GM, Carmichael SL, Yang W, Selvin S, Schaffer DM. Periconceptional dietary intake of choline and betaine and neural tube defects in offspring. *Am J Epidemiol*. 2004;160:102–109.
11. Smithells RW, Sheppard S, Schorah CJ. Vitamin deficiencies and neural tube defects. *Arch Dis Child*. 1976;51:944–950.
12. Mills JL, Tuomilehto J, Yu KF, et al. Maternal vitamin levels during pregnancies producing infants with neural tube defects. *J Pediatr*. 1992;120:863–871.
13. Steen MT, Boddie AM, Fisher AJ, et al. Neural-tube defects are associated with low concentrations of cobalamin (vitamin B12) in amniotic fluid. *Prenat Diagn*. 1998;18:545–555.
14. Wald NJ, Hackshaw AK, Stone R, et al. Blood folic acid and vitamin B12 in relation to neural tube defects. *Br J Obstet Gynaecol*. 1996;103:319–324.
15. Economides DL, Ferguson J, Mackenzie IZ, et al. Folate and vitamin B12 concentrations in maternal and fetal blood, and amniotic fluid in second trimester pregnancies complicated by neural tube defects. *Br J Obstet Gynecol*. 1992;99:23–25.
16. Molloy AM, Kirke P, Hillary I, et al. Maternal serum folate and vitamin B12 concentrations in pregnancies associated with neural tube defects. *Arch Dis Child*. 1985;60:660–665.
17. Croen LA, Shaw GM, Jensvold NJ, Harris JA. Birth defects monitoring in California: a resource for epidemiologic research. *Paediatr Perinat Epidemiol*. 1991;5:423–427.
18. Ueland PM, Midttun O, Windelberg A, Svardal A, Skalevik R, Hustad S. Review: Quantitative profiling of folate and one-carbon metabolism in large-scale epidemiological studies by mass spectrometry. *Clin Chem Lab Med*. 2007;45:1737–1745.
19. Holm PI, Ueland PM, Kvalheim G, Lien EA. Determination of choline, betaine, and dimethylglycine in plasma by a high-throughput method based on normal-phase chromatography-tandem mass spectrometry. *Clin Chem*. 2003;49:286–294.
20. Zeisel SH. Choline: needed for normal development of memory. *J Am Coll Nutr*. 2000;19:528S–531S.
21. Zeisel SH, Blusztajn JK. Choline and human nutrition. *Annu Rev Nutr*. 1994;14:269–296.
22. Shronts EP. Essential nature of choline with implications for total parenteral nutrition. *J Am Diet Assoc*. 1997;97:639–646, 649.
23. Zeisel SH. Nutrigenomics and metabolomics will change clinical nutrition and public health practice: insights from studies on dietary requirements for choline. *Am J Clin Nutr*. 2007;86:542–548.
24. Zeisel SH. Choline, homcysteine, and pregnancy. *Am J Clin Nutr*. 2005;82:719–720.
25. Zeisel SH. Choline: essential for brain development and function. *Adv Pediatr*. 1997;44:263–295.
26. Van den Veyver IB. Genetic effects of methylation diets. *Annu Rev Nutr*. 2002;22:255–282.
27. Dunleavy LPE, Burren KA, Mills K, et al. Integrity of the methylation cycle is essential for mammalian neural tube closure. *Birth Def Res A*. 2006;76:544–552.
28. Fisher MC, Zeisel SH, Mar MH, Sadler TW. Pertubations in choline metabolism cause neural tube defects in mouse embryos in vitro. *FASEB J*. 2002;16:619–622.
29. Fisher MC, Zeisel SH, Mar MH, Sadler TW. Inhibitors of choline uptake and metabolism cause developmental abnormalities in neurulating mouse embryos. *Teratology*. 2001;64:114–122.
30. Wong L, Magdaleno S, Tabas I, Jackowski S. Early embryonic lethality in mice with targeted deletion of the CTP: phosphocholine cytidyltransferase a gene (Pcyt1a). *Mol Cell Biol*. 2005;25:3357–3363.
31. Wu G, Aoyama C, Young SG, Vance DE. Early embryonic lethality caused by disruption of the gene for choline kinase a, the first enzyme in phosphatidylcholine biosynthesis. *J Biol Chem*. 2008;283:1456–1462.
32. Harris MJ, Juriloff DM. Mini-review: toward understanding mechanisms of genetic neural tube defects in mice. *Teratology*. 1999;60:292–305.
33. Petzold A, Stiefel D, Copp AJ. Amniotic fluid brain-specific proteins are biomarkers for spinal cord injury in experimental myelomeningocele. *J Neurochem*. 2005;95:594–598.
34. Zhao W, Mosley BS, Cleves MA, Melnyk S, James SJ, Hobbs CA. Neural tube defects and maternal biomarkers of folate, homocysteine, and glutathione metabolism. *Birth Defects Res A Clin Mol Teratol*. 2006;76:230–236.
35. Mills JL, McPartlin JM, Kirke PN, et al. Homocysteine metabolism in pregnancies complicated by neural-tube defects. *Lancet*. 1995;345:149–151.
36. Van Der Put NM, van Straaten HW, Trijbels FJ, Blom HJ. Folate, homocysteine and neural tube defects: an overview. *Exp Biol Med (Maywood)*. 2001;226:243–270.
37. Adams MJ Jr, Khoury MJ, Scanlon KS, et al. Elevated midtrimester serum methylmalonic acid levels as a risk factor for neural tube defects. *Teratology*. 1995;51:311–317.
38. Ray JG, Wyatt PR, Thompson MD, et al. Vitamin B12 and the risk of neural tube defects in a folic-acid-fortified population. *Epidemiology*. 2007;18:362–366.
39. Lawrence JM, Umekubo MA, Chiu V, Petitti DB. Split sample analysis of serum folate levels after 18 days in frozen storage. *Clin Lab*. 2000;46:483–486.
40. Benevenga NJ. Consideration of betaine and one-carbon sources of N5-methyltetrahydrofolate for use in homocystinuria and neural tube defects. *Am J Clin Nutr*. 2007;85:946–949.