Maternal choline concentrations during pregnancy and choline-related genetic variants as risk factors for neural tube defects[1–3]

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ABSTRACT

Background: Low maternal choline intake and blood concentration may be risk factors for having a child with a neural tube defect (NTD); however, the data are inconsistent. This is an important question to resolve because choline, if taken periconceptionally, might add to the protective effect currently being achieved by folic acid.

Objective: We examined the relation between NTDs, choline status, and genetic polymorphisms reported to influence de novo choline synthesis to investigate claims that taking choline periconceptionally could reduce NTD rates.

Design: Two study groups of pregnant women were investigated: women who had a current NTD-affected pregnancy (AP; $n = 71$) and unaffected controls ($n = 214$) and women who had an NTD in another pregnancy but not in the current pregnancy [nonaffected pregnancy (NAP); $n = 98$] and unaffected controls ($n = 386$). Blood samples to measure betaine and total choline concentrations and single nucleotide polymorphisms related to choline metabolism were collected at their first prenatal visit.

Results: Mean (±SD) plasma total choline concentrations in the AP (2.8 ± 1.0 mmol/L) and control (2.9 ± 0.9 mmol/L) groups did not differ significantly. Betaine concentrations were not significantly different between the 2 groups. Total choline and betaine in the NAP group did not differ from controls. Cases were significantly more likely to have the G allele of phosphatidylethanolamine-*N*-methyltransferase (*PEMT*; V175M, +5465 G>A) rs7946 ($P = 0.02$).

Conclusions: Our results indicate that maternal betaine and choline concentrations are not strongly associated with NTD risk. The association between *PEMT* rs7946 and NTDs requires confirmation. The addition of choline to folic acid supplements may not further reduce NTD risk. *Am J Clin Nutr* 2014;100:1069–74.

INTRODUCTION

Low maternal dietary choline intake and low serum total choline concentrations have been reported to be risk factors for neural tube defects (NTDs)[4] (1, 2). Two recent publications, however, showed no association between maternal choline intake and NTD risk (3, 4). This is an important question to resolve because choline, if taken periconceptionally, might add to the protective effect currently being achieved by folic acid. The American College of Medical Genetics (5) stated that "replication [of the positive] studies should be done before proceeding with food fortification with choline." Furthermore, because both choline and folate are involved in methylation, confirming a beneficial effect of choline could help elucidate the mechanism by which folate prevents NTDs.

The phosphatidylethanolamine-*N*-methyltransferase (*PEMT*) gene encodes the only enzyme known to produce choline de novo in mammals by the triple methylation of phosphatidylethanolamine to produce phosphatidylcholine. Several single nucleotide polymorphisms (SNPs) in the *PEMT* gene were reported to alter choline homeostasis (6–8). *PEMT* rs7946 is a missense mutation, V175M (+5465G>A) in exon 8 of the gene, in which homocysteine concentrations were reported to increase when folate was restricted (7). A second SNP, *PEMT* rs12325817, has been linked to hepatic steatosis (8) in those who have inadequate choline intake. A third SNP, *PEMT* rs897453, which also produces a nonsynonymous mutation (V581I), has been associated with altered risk for NTDs in conjunction with *PEMT* rs7946 (9). Furthermore, in a recent study of 1441 SNPs in 82 candidate genes, *PEMT* was one of the genes that carried the 10 strongest association signals (10).

We measured total choline and betaine concentrations in pregnant women who were currently undergoing a pregnancy with an NTD-affected fetus [affected pregnancy (AP)], pregnant women who had other pregnancies affected by NTD [nonaffected pregnancy (NAP)], and pregnant women carrying unaffected fetuses to address the following question: Is low serum choline a risk factor for NTDs? We also examined several important SNPs in the *PEMT* gene.

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[4] Abbreviations used: AP, affected pregnancy; NAP, nonaffected pregnancy; NTD, neural tube defect; SNP, single nucleotide polymorphism.

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SUBJECTS AND METHODS

Between 1986 and 1990, with ethical approval, research blood samples were collected from 56,049 nonfasting women at their first antenatal clinic visit in the 3 major Dublin hospitals. This represents ~70% of women who delivered in these hospitals during that time period. Further details on sample collection and processing were published elsewhere (11–13). In brief, blood samples were collected into dipotassium EDTA-containing tubes. Samples were kept at room temperature until they were transferred to the laboratory at 4°C within 1 to 2 hours after drawing. Batchwise processing of plasma was carried out on a daily basis for all samples collected. Samples were logged and processed in chronologic order. An aliquot in 1% ascorbic acid for red blood cell folate, a plasma sample in a tube containing EDTA, and a whole blood sample were stored below –20°C for each participant. Betaine and total choline concentrations were assayed in 2008.

Details of participant and sample information for the AP and the NAP groups were published elsewhere (14). Briefly, information was available on 84 women who had an NTD-AP (AP cases) and 266 control subjects from unaffected pregnancies (AP controls). For the current study, 8 of the 84 AP case mothers had no blood sample remaining and 5 used vitamin supplements, leaving 71 for analysis. Of the 266 controls, 28 had no blood samples available and 25 had taken supplements including one who was also missing the blood sample, leaving 214 for analysis.

From the European Surveillance of Congenital Anomalies Network birth defects registry and hospital registers we ascertained that 303 women with a history of NTD-affected birth had one or more pregnancies that were not affected by NTD (NAP) between 1986 and 1990 in these 3 hospitals. Data were available on 125 NAP cases. Of these, 13 had no blood available and 15 took supplements including one who was also missing the blood sample, leaving 98 for analysis. Controls (NAP controls) were obtained for each NAP case subject by selecting 4 or 5 women who attended the antenatal clinic in the same hospital on the same day. Hospital charts for these women were then scrutinized for supplementation status and medical history. From this, 462 women were selected. For the current study, 43 had no blood available and 35 were excluded because of supplement use, including 2 who were missing blood samples, leaving 386 for analysis. All samples were made anonymous before analysis. The study was approved by the institutional review board at the National Human Genome Research Institute, NIH.

Laboratory methods

Total choline, along with the oxidative product betaine, were measured on plasma samples by using a high-throughput normal-phase chromatography tandem mass spectrometry method (15). Total homocysteine was assayed by methylchloroformate derivatization and gas chromatography–mass spectrometry (16). Red blood cell folate was measured by microbiological assay, as previously described (17).

SNP selection and genotyping

The strategy for SNP selection was as follows. *MTHFR* C677T and *MTHFD1* R653Q were included because we had identified them as NTD risk factors in this population. *PEMT* was chosen as the target gene because it is the only enzyme that produces choline de novo in humans. We chose specific *PEMT* SNPs on the basis of reports that they *1*) altered choline homeostasis, eg, increasing total homocysteine, producing hepatic steatosis, or altering choline metabolism, or *2*) changed NTD risk.

The extraction of genomic DNA from whole blood samples was performed by using the QIAamp DNA Blood Mini Kit (Qiagen). Genotypes were generated on the selected SNPs by KASPar chemistry at LGC Genomics. A quality-control sample was included on each plate of 100 samples, and 5% of samples were duplicated at random across all plates, which were blinded to analysts. The overall call rate was 97.4% for rs9743, 98.7% for rs897453, and 96.3% for rs12325817. Concordance rates for quality control and duplicate samples were >99.99% for the 3 SNPs.

Statistical methods

Demographic data for NTD cases and controls in the AP and NAP studies were compared by using chi-square tests. The choline data distribution was not compatible with a standard parametric distribution, nor could it be transformed to normal by log or Box-Cox transformations. Therefore, nonparametric permutation *t* tests and permutation Kruskal-Wallis tests were used to compare the choline concentration means and medians between cases and controls, and these were also performed within groups for each type of NTD and quartile of red blood cell folate for total choline and betaine. Associations between variables were determined by using the Spearman correlation coefficient. Associations between NTDs and SNPs in the enzyme genes were examined by using chi-square tests. Logistic regression models were fitted to test the association of NTDs with total choline, betaine, homocysteine, and *PEMT* gene SNP rs7946 in conjunction with rs897453 with the use of maternal age and year of sample as covariates. Statistical analyses were performed with SAS version 9.3 (SAS Institute).

RESULTS

Samples were analyzed for 71 AP cases, 214 women whose fetuses had no major malformations (AP controls), 98 NAP cases, and 386 women whose fetuses had no major malformations (NAP controls). The median gestational age at time of sampling was 14.4 wk in the AP cases, 15.1 wk in the AP controls, 15.3 wk in the NAP cases, and 14.3 wk in the NAP controls. The AP case mothers were significantly younger ($P = 0.03$) than AP control mothers (**Table 1**). They were less likely to have had a live birth ($P = 0.02$) and were of lower parity ($P = 0.03$). They were much more likely to deliver preterm ($P < 0.0001$). They did not differ in number of pregnancy losses, marital status, or the year of sample collection.

In large part because the majority of NAP cases had NTD offspring before the study pregnancy, NAP cases differed from NAP controls: cases were older, more likely to be married, and had more prior pregnancies and prior live births (all $P < 0.0001$) (*see* Table 1). They did not differ in gestational age at delivery or year of sample collection.

The mean (±SD) total choline concentration (**Table 2**) in the AP cases (2.8 ± 1.0 mmol/L) was not significantly different from that in the AP controls (2.9 ± 0.9 mmol/L). The mean betaine concentration in the AP cases (15.4 ± 6.2 μmol/L) also did not differ significantly from that in the AP controls (15.2 ± 5.6 μmol/L). When the analysis was repeated examining spina bifida ($n = 36$)

TABLE 1
Maternal pregnancy history of women with NTD-APs and women with NAPs and their respective controls[1]

	NTD-AP			NAP		
	Cases (n = 71)	Controls (n = 214)	Total (n = 285)	Cases (n = 98)	Controls (n = 386)	Total (n = 484)
	n (%)	*n (%)*	*n*	*n (%)*	*n (%)*	*n*
Maternal age at delivery						
<20 y	7 (9.86)	6 (2.8)	13	0 (0.00)	17 (4.42)	17
20–24 y	21 (29.58)	54 (25.23)	75	9 (9.28)	95 (24.68)	104
25–29 y	15 (22.37)	75 (35.05)	94	25 (25.77)	133 (34.55)	158
30–34 y	20 (28.17)	48 (22.43)	68	34 (35.05)	95 (24.68)	129
≥35 y	8 (11.27)	31 (14.49)	39	29 (29.9)	45 (11.69)	74
Total	71	214	285	97	385	482
Missing		0			2	
P		0.03			<0.0001	
Prior pregnancies						
0	37 (52.11)	71 (33.18)	108	0 (0.00)	119 (30.83)	119
1	12 (16.90)	51 (23.83)	63	5 (5.15)	103 (26.68)	108
2	5 (7.04)	40 (18.69)	45	15 (15.46)	59 (15.28)	74
3	6 (8.45)	19 (8.88)	25	23 (23.71)	47 (12.18)	70
≥4	11 (15.49)	33 (15.42)	44	54 (55.67)	58 (15.03)	112
Total	71	214	285	97	386	483
Missing		0			1	
P		0.03			<0.0001	
Prior live births						
0	39 (54.93)	74 (34.58)	113	3 (3.09)	15 (5.62)	18
1	11 (15.49)	53 (24.77)	64	14 (14.43)	96 (35.96)	110
2	6 (8.45)	43 (20.09)	49	26 (26.80)	71 (26.59)	97
3	8 (11.27)	20 (9.35)	28	19 (19.59)	43 (16.10)	62
≥4	7 (9.86)	24 (11.21)	31	35 (26.08)	42 (15.73)	77
Total	71	214	285	97	267	364
Missing		0			120	
P		0.02			<0.0001	
Prior losses						
0	70 (98.59)	213 (99.53)	283	53 (54.64)	259 (97.00)	312
1	1 (1.41)	1 (0.47)	2	41 (42.27)	8 (3.00)	49
≥2	0 (0.00)	0 (0.00)	0	3 (3.09)	0 (0.00)	3
Total	71	214	285	97	267	364
Missing		0			120	
P		0.41			<0.0001	
Gestational age at delivery						
≤12 wk	0 (0.00)	0 (0.00)	0	2 (2.06)	6 (1.56)	8
13–29 wk	3 (4.23)	2 (0.93)	5	1 (1.03)	9 (2.34)	10
30–36 wk	21 (29.58)	7 (3.27)	28	8 (8.25)	17 (4.42)	25
≥37 wk	47 (66.20)	205 (95.79)	252	86 (88.66)	353 (91.69)	439
Total	71	214	285	97	385	482
Missing		0			2	
P		<0.0001			0.39	
Maternal marital status						
Married	52 (73.24)	173 (80.84)	225	93 (100.00)	308 (80.84)	401
Single	18 (25.35)	38 (17.76)	56	0 (0.00)	73 (19.16)	73
Other	1 (1.41)	3 (1.40)	4	0 (0.00)	0 (0.00)	0
Total	71	214	285	93	381	474
Missing		0			10	
P		0.38			<0.0001	

[1] Demographic data for AP and NAP NTD cases and controls were compared by using chi-square tests. AP, affected pregnancy; NAP, nonaffected pregnancy; NTD, neural tube defect.

and anencephaly (n = 29) cases separately, neither total choline nor betaine concentrations differed significantly in the spina bifida or anencephaly case mothers compared with controls.

In the NAP cases, mean and median total choline and betaine concentrations did not differ significantly from concentrations in NAP controls (Table 2). When the NAP cases were divided by type of NTD, there were no significant differences between the NAP NTD groups and their controls in total choline or betaine concentrations. Adjustment for maternal age and date of sample did not change the results appreciably.

To determine how folate status affected the relation between choline and NTDs, we divided the groups into quartiles by control

TABLE 2
Total choline, betaine, and homocysteine concentrations in women with APs, women who had NAPs, and their respective control groups[1]

	Total choline (mmol/L)			Betaine (μmol/L)			Homocysteine (μmol/L)		
	n	Median (IQR)	Mean ± SD	*n*	Median (IQR)	Mean ± SD	*n*	Median (IQR)	Mean ± SD
AP									
Cases	71	2.6 (1.5)	2.8 ± 1.0	71	13.5 (6.8)	15.4 ± 6.2	71	9.8 (3.4)	10.5 ± 3.1
Controls	214	2.9 (1.3)	2.9 ± 0.9	214	14.0 (6.1)	15.2 ± 5.6	214	8.6 (3.2)	9.5 ± 3.4
Permutation *P* value		0.30[2]	0.31[3]		0.90[2]	0.89[3]		0.002[2]	0.04[3]
NAP									
Cases	98	2.5 (1.0)	2.6 ± 0.7	98	14.9 (7.2)	15.8 ± 6.3	98	10.1 (4.7)	10.7 ± 3.6
Controls	386	2.4 (0.9)	2.6 ± 0.7	386	14.9 (6.9)	16.2 ± 6.6	386	9.3 (2.8)	9.6 ± 2.6
Permutation *P* value		0.71[2]	0.96[3]		0.48[2]	0.57[3]		0.003[2]	0.0003[3]

[1] AP, affected pregnancy; NAP, nonaffected pregnancy.
[2] Comparison of medians by permutation Kruskal-Wallis test.
[3] Comparison of means by permutation *t* test.

group red blood cell folate concentrations (**Figure 1**). The AP case group did not have significantly different values for total choline or betaine compared with AP controls in any of the folate quartiles. The NAP case group had significantly higher mean total choline concentrations in the third quartile compared with the NAP control group. These findings could be due to chance because there were no consistent differences between groups in the other quartiles.

Homocysteine was previously reported to be higher in AP cases than in controls (13). We have not previously analyzed homocysteine in relation to NAP pregnancies. The current analysis (Table 2) showed that it was also significantly higher in the NAP cases (10.7 ± 3.6 μmol/L) than in the NAP controls (9.6 ± 2.6 μmol/L; $P = 0.0003$).

We examined 3 SNPs in the *PEMT* gene that were reported to affect choline metabolism (**Table 3**). *PEMT* V175M (rs7946) genotypes were significantly different in the combined AP and NAP case mothers compared with the combined control groups ($P = 0.03$). Cases were more likely to have the rarer *GG* genotype (9.40% compared with 4.87%, respectively). Total choline and betaine concentrations did not differ significantly by allele (data not shown). The case and control genotypes did not differ significantly for the other *PEMT* SNPs (Table 3). We also examined total choline and betaine concentrations in relation to *MTHFR* C677>T (rs1801133) and *MTHFD1* R653Q (1958 G>A) (rs2236225), variants (Table 3) that we previously reported to be associated with NTDs (18–21). Total choline and betaine concentrations did not differ between those who were homozygous for the rare variants (*TT* in *MTHFR* and *AA* in *MTHFD1*) and those who were not. Genotype was not related to homocysteine concentration in the 3 *PEMT* SNPs or in *MTHFD1* R563Q. As noted previously, *MTHFR* C677>T was strongly associated with homocysteine, which increased with each additional T allele ($P < 0.0001$). Examining the effect of the *PEMT* SNPs after adjustment for *MTHFR 677* genotype showed no significant association with homocysteine concentration.

DISCUSSION

Our data showed no significant relation between blood choline concentration and NTDs. No difference in total choline or betaine concentrations was seen between AP or NAP cases and their respective controls.

Previous studies have reported lower total choline intake and serum total choline concentrations in women with NTD pregnancies compared with those with unaffected pregnancies (1, 2). A recent study from the same group (3) and one from the National

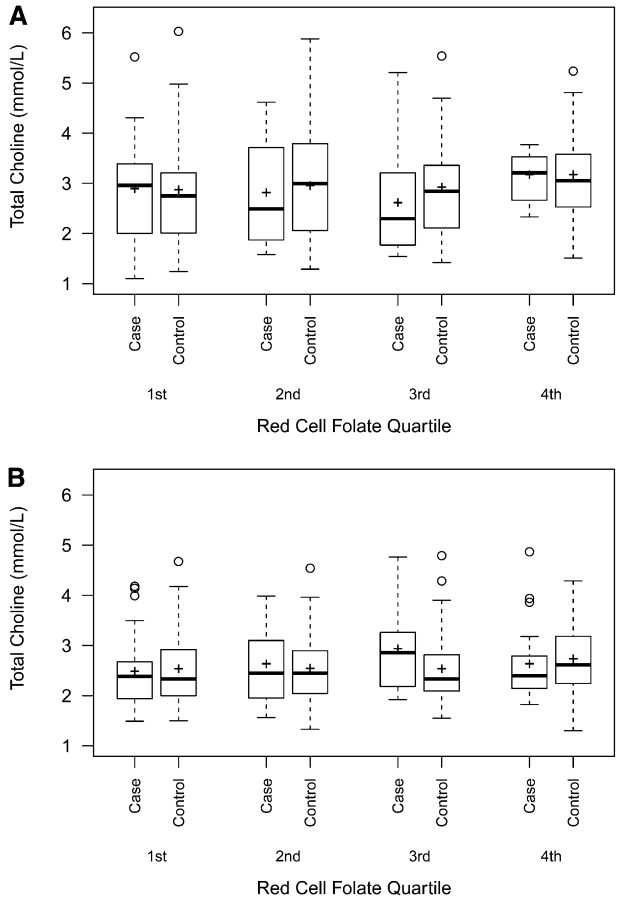

FIGURE 1. Choline concentrations in cases and controls by quartile of control red blood cell folate concentration. A: Total choline, affected pregnancy groups. B: Total choline, nonaffected pregnancy groups. Mean total concentrations were significantly higher in cases than in controls in the third quartile of the nonaffected pregnancy group. The bottom and top edges of the boxes are located at the 25th and 75th percentiles. Center horizontal lines represent medians. Central plus signs (+) represent means. Central vertical lines extend as far as the data do, to a distance of not more than 1.5 IQRs. Zeroes indicate a value within 3 IQRs of the box.

TABLE 3
Genotype data for the combined NTD case group and combined control group[1]

	Cases	Controls	Total	*P*
PEMT rs7946				
n	149	493	642	
GG	14 (9.4)	24 (4.87)	38	0.03
GA	56 (37.58)	157 (31.85)	213	
AA	79 (53.02)	312 (63.29)	391	
AA	79 (53.02)	312 (63.29)	391	0.02
Non-AA	70 (46.98)	181 (36.71)	251	
PEMT rs897453				
n	148	502	650	
AA	34 (22.97)	111 (22.11)	145	0.53
GA	67 (45.27)	252 (50.20)	319	
GG	47 (31.76)	139 (27.69)	186	
AA	34 (22.97)	111 (22.11)	145	0.82
Non-AA	114 (77.03)	391 (77.89)	505	
PEMT rs12325817				
n	145	486	631	
CC	44 (30.34)	140 (28.80)	184	0.53
GC	72 (49.66)	227 (46.71)	299	
GG	29 (20.00)	119 (24.49)	148	
GG	29 (20.00)	119 (24.49)	148	0.26
Non-GG	116 (80.00)	367 (75.51)	483	
*MTHFR*_C677T rs1801133				
n	138	493	631	
CC	56 (40.58)	222 (45.03)	278	0.25
CT	62 (44.93)	223 (45.23)	285	
TT	20 (14.49)	48 (9.74)	68	
CC	56 (40.58)	222 (45.03)	278	0.35
Non-CC	82 (59.42)	271 (54.97)	353	
MTHFD1 R653Q rs2236225				
n	146	488	634	
GG	33 (22.60)	133 (27.26)	166	0.11
GA	72 (49.32)	257 (52.66)	329	
AA	41 (28.08)	98 (20.08)	139	
AA	41 (28.08)	98 (20.08)	139	0.04
Non-AA	105 (71.92)	390 (79.92)	494	

[1] All values are *n* (%) unless otherwise indicated. NTD cases and controls were compared by using chi-square tests. NTD, neural tube defect.

Birth Defects Prevention Study (4), however, did not show a significant difference in choline intake. Betaine has received less attention than choline. Apart from its endogenous production from choline, betaine is obtained from dietary sources, particularly from grain (22). Chandler et al (4) reported that betaine intake was lower in mothers of children with anencephaly but not spina bifida. We found no difference in betaine concentrations between mothers of children with either anencephaly or spina bifida and control mothers.

The G allele of *PEMT* gene SNP rs7946 was significantly associated with NTDs in our combined population of mothers. Our data indicating that *PEMT* may be associated with NTD risk are supported by our earlier work on candidate genes in which 3 different *PEMT* SNPs showed associations with NTDs (10). One other study of rs7946 found that it alone was not a risk factor for NTDs but that homozygosity for the other (*AA*) genotype was a risk in conjunction with rs897453 (9). We did not find an interaction between the 2 SNPs. The effect of genetic variants could depend on choline status. Thus, in a population with poorer choline intake, additional associations might be found.

It should be noted, however, that the *PEMT* rs7946 G allele did not change homocysteine concentrations significantly. Therefore, it remains to be determined how it could affect NTD risk. It is also worth noting that, despite our large study population, we did not see associations between any of the 3 *PEMT* SNPs we studied on homocysteine, betaine, or choline concentrations. *MTHFR* 677TT showed a very strong effect on homocysteine, and adjustment for *MTHFR* 677TT did not alter the finding of no association with the *PEMT* SNPs.

Our study had several strengths. Samples were obtained from a large number of women carrying affected fetuses at a time when supplement use and food fortification were very uncommon, and we were able to exclude supplement takers. We were also able to account for some of the genetic differences in choline production by evaluating important polymorphisms in *PEMT*. Some limitations should also be noted. We were not able to collect dietary data; we had limited data on sociodemographic factors; and we could not identify teratogens such as drugs that could have caused some of the NTDs, although teratogens generally account for only a small proportion of NTDs. Plasma total choline is not an exact indicator of dietary choline intake because plasma phosphatidylcholine, the major component of plasma choline, is influenced by plasma lipoprotein concentrations. The other limitation is that the sample size for this investigation was somewhat smaller than our previous reports because of sample depletion. For this reason, the association between NTD and *MTHFR* 677T (18–21) was no longer significant.

Our finding that *PEMT* rs7946 was associated with NTD risk requires confirmation, because it was not associated with a change in choline, betaine, or homocysteine. From the clinical perspective, women of childbearing age are advised to take folic acid routinely to ensure that their folate status is adequate should they become pregnant (23). Our data suggest that adding choline is not likely to enhance the protective effect of folic acid in a population such as ours in whom supplement use and fortified food exposure are rare.

The authors' responsibilities were as follows—JLM, LCB, PNK, and AMM: were involved in the design of the study; AMM, PMU, PNK, and LCB: were involved in the generation of data for the study; JLM, RF, AL, YW, AMM, and BS: were involved in the analysis of the data; JLM, PMU, BS, LCB, RF, YW, and AMM: were involved in producing the report; and JLM had primary responsibility for the final content. All of the authors read and approved the final manuscript. The authors had no conflicts of interest to declare.

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