Association between serum folate and vitamin B-12 and outcomes of assisted reproductive technologies[1]

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ABSTRACT

Background: Preconceptional folate and vitamin B-12 have been linked to beneficial reproductive outcomes in both natural pregnancies and those after assisted reproductive technology (ART) treatment.

Objective: The objective of the study was to evaluate the associations of serum folate and vitamin B-12 with ART outcomes.

Design: This analysis included a random sample of 100 women (154 ART cycles) participating in a prospective cohort study [Environment and Reproductive Health (EARTH)] at the Massachusetts General Hospital Fertility Center (2007–2013). Serum folate and vitamin B-12 were measured in blood samples collected between days 3 and 9 of treatment. Generalized estimating equations with adjustment for age, BMI, and race were used to evaluate the association of serum folate and vitamin B-12 with ART outcomes.

Results: Women in the highest quartile of serum folate (>26.3 ng/mL) had 1.62 (95% CI: 0.99, 2.65) times the probability of live birth compared with women in the lowest quartile (<16.6 ng/mL). Women in the highest quartile of serum vitamin B-12 (>701 pg/mL) had 2.04 (95% CI: 1.14, 3.62) times the probability of live birth compared with women in the lowest quartile (<439 pg/mL). Suggestive evidence of an interaction was observed; women with serum folate and vitamin B-12 concentrations greater than the median had 1.92 (95% CI: 1.12, 3.29) times the probability of live birth compared with women with folate and vitamin B-12 concentrations less than or equal to the median. This translated into an adjusted difference in live birth rates of 26% (95% CI: 10%, 48%; $P = 0.02$).

Conclusion: Higher serum concentrations of folate and vitamin B-12 before ART treatment were associated with higher live birth rates among a population exposed to folic acid fortification. This trial was registered at clinicaltrials.gov as NCT00011713. *Am J Clin Nutr* 2015;102:943–50.

Keywords: assisted reproduction, folate, infertility, vitamin B-12, pregnancy, in vitro fertilization

INTRODUCTION

Infertility, defined as the inability to conceive after 12 mo of unprotected intercourse, is a common reproductive disorder affecting ~15% of couples who attempt to become pregnant (1). Assisted reproductive technologies (ARTs),[8] which include in vitro fertilization and intracytoplasmic sperm injection, have become the main treatment modalities for couples facing infertility (2). Since 2007, nearly 150,000 ART cycles are performed yearly in the United States, which accounts for ~2% of live births nationwide (3, 4). In Europe, more than half a million ART cycles were performed in 2010, accounting for 1.7–5.9% of live births depending on the specific European country (5). Despite major advances in infertility treatments, live birth rates per initiated cycle have remained constant at ~30% since 2002 (4), which highlights the need to identify modifiable predictors of successful infertility treatment with ART. Whereas research on dietary modification before ART treatment is sparse, there is reason to believe that certain micronutrients such as folate and vitamin B-12 could positively influence reproductive success.

Studies among couples undergoing infertility treatment in Europe suggest that folate may improve total and mature oocyte counts (6), embryo quality (7), and pregnancy rates (8). However, in studies that investigated clinical outcomes of infertility treatment solely among women undergoing embryo transfer, no associations with live birth were observed (9–11). Several small studies and case reports have found associations between vitamin B-12 deficiency and female subfertility (12–14). Moreover, a cohort study from the Netherlands found that vitamin B-12 concentrations in serum and follicular fluid were positively correlated with embryo quality (7). A large cohort study from the United Kingdom found no relation between plasma folate and vitamin B-12 concentrations and clinical outcomes of in vitro fertilization among women undergoing embryo transfer; however, there was a significantly higher rate of twin births in

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[8] Abbreviations used: ART, assisted reproductive technology; DFE, dietary folate equivalent; EARTH, Environment and Reproductive Health; GnRH, gonadotropin-releasing hormone.

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women with higher plasma folate and vitamin B-12 concentrations, which suggests that these B vitamins might increase the likelihood of each potentially viable embryo giving rise to a live birth (10).

Previously, we reported that pretreatment intakes of folate and vitamin B-12 were related to a higher probability of live birth among women undergoing ART in the United States (15). Whereas our results were intriguing, our assessment of folate and vitamin B-12 was limited to self-reported dietary assessment with a food-frequency questionnaire. To expand on our previous findings, we evaluated the relation between serum folate and vitamin B-12 concentrations and infertility treatment outcomes among women undergoing ART at an academic medical center in the United States.

METHODS

Study population

Participants were a random sample of women enrolled in the Environment and Reproductive Health (EARTH) Study—an ongoing prospective cohort started in 2006 to identify determinants of fertility among couples presenting to the Massachusetts General Hospital Fertility Center (Boston, Massachusetts). Women were eligible for this analysis if they had completed a food-frequency questionnaire (introduced in 2007) and had subsequently completed at least one ART cycle by May 2013 (n = 232). From this initial pool of 232 women, we randomly selected 100 women (contributing 154 ART cycles) to have their stored blood samples sent for analysis of folate and vitamin B-12. The study was approved by the Institutional Review Boards of the Massachusetts General Hospital and the Harvard School of Public Health. All participants provided written informed consent after the study procedures were explained by a research nurse.

Biospecimen collection and assessment

Blood samples were collected from women between days 3 and 9 of gonadotropin treatment during their first in-study ART cycle. Serum concentrations of folate and vitamin B-12 were measured at the Clinical & Epidemiologic Research Laboratory at Boston Children's Hospital. Serum folate was measured by using an electrochemiluminescence binding assay on the Roche E Modular system (Roche Diagnostics) (16). The lowest detection limit of this assay is 0.6 ng/mL, and the day-to-day imprecision values at concentrations of 7.6, 14.3, and 19.2 ng/mL are 3.9%, 3.1%, and 2.0%, respectively. Serum vitamin B-12 was measured by an electrochemiluminescence immunoassay technique on the Roche E Modular system (Roche Diagnostics) (16). The lowest detection limit of this assay is 30 pg/mL, and the day-to-day imprecision values at concentrations of 203, 481, and 1499 pg/mL are 7.6%, 4.4%, and 3.2%, respectively. These are both Food and Drug Administration–approved clinical assays. We defined serum folate and vitamin B-12 deficiencies as $<$4 ng/mL (17) and $<$200 pg/mL (18), respectively.

Dietary assessment

Diet was assessed before ART treatment by using a validated food-frequency questionnaire (19). Participants were asked to report how often they consumed specified amounts of 131 food items during the previous year. Multivitamin and supplement users were asked to specify the brand of the multivitamin or supplement, the dose, and frequency of use. Nutrient intakes were estimated by summing the nutrient contribution of all food and supplement items. Nutrient contents were obtained from the nutrient database of the US Department of Agriculture with additional information from manufacturers (20). Dietary folate equivalents (DFEs) were calculated to account for differences in absorption between natural and synthetic folate (21).

Covariate assessment

At enrollment into the EARTH Study, height and weight were measured by a trained research nurse to calculate BMI (in kg/m^2), and a brief, nurse-administered questionnaire was used to collect data on demographic factors, medical history, and lifestyle. Participants also completed a detailed take-home questionnaire with additional questions on lifestyle factors, reproductive health, and medical history. Clinical information, including infertility diagnosis and protocol type, was abstracted from electronic medical records.

Clinical procedures and outcome assessment

Patients underwent 1 of 3 stimulation protocols as clinically indicated: *1*) luteal-phase gonadotropin-releasing hormone (GnRH) agonist protocol, *2*) follicular-phase GnRH-agonist/flare protocol; or *3*) follicular-phase GnRH-antagonist protocol. Patients were monitored during gonadotropin stimulation for serum estradiol, follicle size measurements and counts, and endometrial thickness through 2 d before egg retrieval. Human chorionic gonadotropin was administered $\sim$36 h before the scheduled egg-retrieval procedure to induce ovulation. Couples underwent ART with conventional in vitro fertilization or intracytoplasmatic sperm injection as clinically indicated.

Embryologists classified oocytes as germinal vesicle, metaphase I, metaphase II, or degenerated. Embryologists determined the fertilization rate 17–20 h after insemination as the number of oocytes with 2 pronuclei divided by the number of inseminated metaphase II oocytes. The resulting embryos were monitored for cell number and morphologic quality [1 (best) to 5 (worst)] on days 2 and 3. For analysis, we classified embryos as best quality if they had 4 cells on day 2, 8 cells on day 3, and a morphologic quality score of 1 or 2 on days 2 and 3. We defined implantation as a serum β-human chorionic gonadotropin concentration $>$6 mIU/mL typically measured 17 d (range: 15–20 d) after egg retrieval, clinical pregnancy as the presence of an intrauterine pregnancy confirmed by ultrasonography, and live birth as the birth of a neonate on or after 24 wk of gestation.

Statistical analysis

Pearson's correlation coefficient was used to describe the measure of dependence between serum concentrations and dietary intake as well as between serum folate and vitamin B-12 concentrations. Serum folate and vitamin B-12 concentrations were log transformed for these analyses to better approximate a normal distribution. Women were classified into quartiles based on serum folate and vitamin B-12 concentrations. Descriptive statistics were calculated for demographic, reproductive, and dietary characteristics according to these quartiles. In addition

to assessing serum folate and vitamin B-12 as independent measures, we cross-classified women into 4 categories based on joint levels of high (greater than the median) and low (less than or equal to the median) serum folate and vitamin B-12 concentrations to evaluate possible joint effects and possible interactions. We decided a priori to present results for this subanalysis due to biological rationale, recognizing the low statistical power to detect this interaction. Serum folate and vitamin B-12 were also analyzed as continuous, linear variables.

Generalized estimating equations were used to evaluate the association between serum folate and vitamin B-12 concentrations and ART outcomes while accounting for within-person correlations in outcomes. A Poisson distribution and log link function were specified for oocyte counts, and a binomial distribution and log link function were specified for fertilization, embryo quality, and clinical outcomes. Tests for trend across quartiles were conducted by using a variable with the median serum concentration in each quartile as a continuous variable. Results are presented as RRs and 95% CIs from a comparison of quartiles 2, 3, and 4 with quartile 1 or as population marginal means, adjusted for covariates.

Confounding was evaluated by using prior knowledge and descriptive statistics from our cohort. The following covariates were considered for inclusion in the final model: age (continuous), BMI (continuous), smoking status (ever smoked and never smoked), race (white and other), and primary infertility diagnosis (female factor, male factor, and unexplained). In addition, treatment protocol type (luteal phase or follicular phase GnRH agonist/GnRH antagonist) was evaluated as both an intermediate and confounding variable. Variables were retained in the final model if they changed the regression coefficient for the primary exposure by $\geq$10%. We conducted all statistical analyses using SAS version 9.4 (SAS Institute Inc.) and considered 2-sided significance levels $<$0.05 as statistically significant.

RESULTS

The 100 women had a median calorie-adjusted folate intake of 1971 μg/d in DFE (range: 375–4012 μg DFE/d) and a median calorie-adjusted vitamin B-12 concentration of 12 μg/d (range: 4–585 μg/d). Serum concentrations of folate and vitamin B-12 were modestly correlated with dietary intake (ρ = 0.27 and 0.22, respectively) and with each other (ρ = 0.24). The distributions of serum folate and vitamin B-12 in this population are shown in **Figure 1**. No women in our cohort had deficient serum folate concentrations, and only 3 women had deficient serum vitamin B-12 concentrations (18). Women were followed for 1 (66%), 2 (21%), 3 (8%), 4 (3%), or 5 (2%) ART cycles. The median time

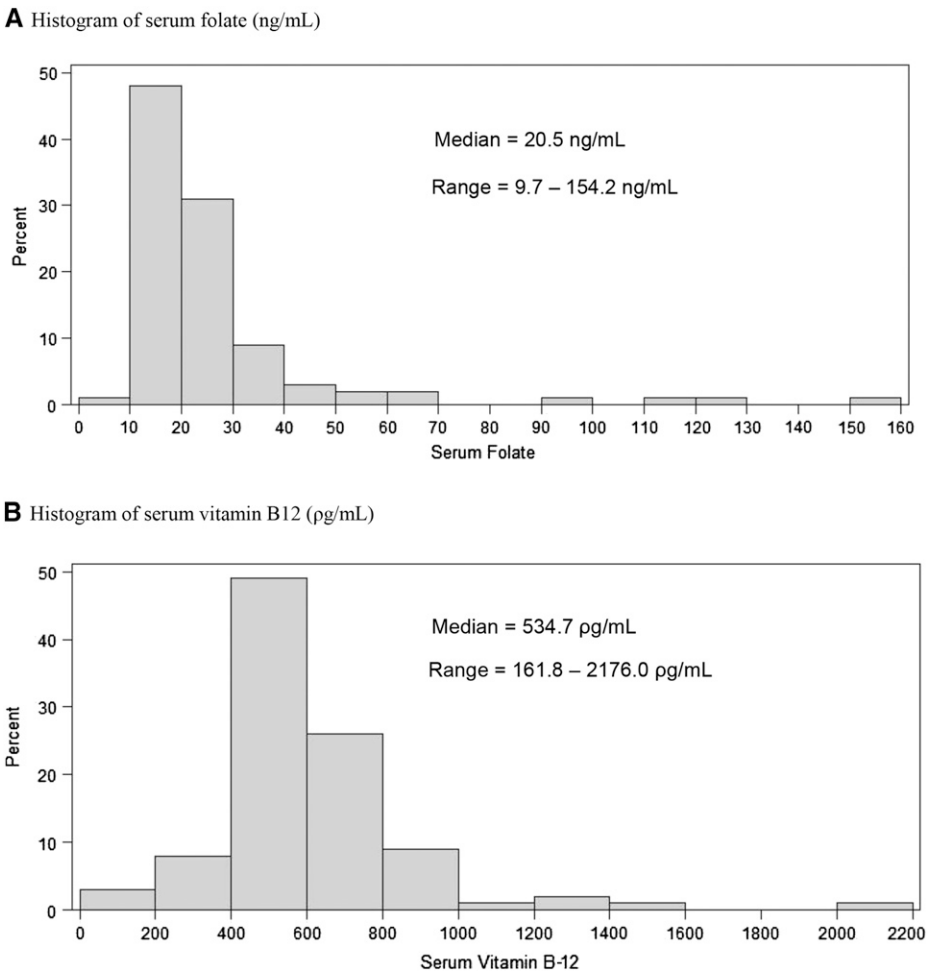

FIGURE 1 Distribution of serum folate (A) and vitamin B-12 (B) concentrations in 100 women from the Environment and Reproductive Health Study (2007–2013).

between blood draw and start of the last ART cycle was 187 d. Baseline demographic, reproductive, and dietary characteristics were generally similar across quartiles of serum folate and vitamin B-12 (**Table 1**), although women in the highest quartile of serum vitamin B-12 had significantly lower BMIs than did women in the lowest quartile.

Women with higher serum folate concentrations had significantly higher clinical pregnancy and live-birth rates after multivariable adjustment (P-trend = 0.04 and 0.01, respectively) (**Table 2**). Specifically, women in the highest quartile of serum folate (>26.3 ng/mL) had 1.50 (95% CI: 0.98, 2.32) times the probability of clinical pregnancy and 1.62 (95% CI: 0.99, 2.65) times the probability of live birth after ART treatment compared with women in the lowest quartile (<16.6 ng/mL). Similarly, women with higher serum vitamin B-12 concentrations had significantly higher implantation, clinical pregnancy, and live-birth rates after multivariable adjustment (P-trend = 0.03, 0.01, and 0.008, respectively). Women in the highest quartile of serum vitamin B-12 (>701 pg/mL) had 1.31 (95% CI: 0.95, 1.80) times the probability of implantation, 1.43 (95% CI: 0.98, 2.08) times the probability of clinical pregnancy, and 2.04 (95% CI: 1.14, 3.62) times the probability of live birth after ART treatment compared with women in the lowest quartile (<439 pg/mL). These results were consistent when serum folate and vitamin B-12 were analyzed on a continuous level; the RRs for live birth were 1.14 (95% CI: 1.08, 1.21) per 20 ng/mL serum folate and 1.13 (95% CI: 1.03, 1.23) per 200 pg/mL serum vitamin B-12.

TABLE 1
Baseline characteristics of 100 women from the Environment and Reproductive Health Study (2007–2013) by quartile range of serum folate and vitamin B-12[1]

	Total cohort	Serum folate, ng/mL		Serum vitamin B-12, pg /mL	
		Q1 (<16.6)	Q4 (>26.5)	Q1 (<438)	Q4 (>701)
No. of subjects	100	25	25	25	25
Personal characteristics					
Age, y	34.7 ± 3.8[2]	34.8 ± 4.5	34.2 ± 3.9	34.5 ± 4.0	33.8 ± 3.9
BMI, kg/m^2	24.3 ± 3.9	25.8 ± 5.5	24.0 ± 2.7	27.2 ± 5.2	22.8 ± 2.8*
Ever smoker, *n* (%)	29 (29.0)	4 (16.0)	7 (28.0)	7 (28.0)	6 (24.0)
White/Caucasian, *n* (%)	80 (80.0)	21 (84.0)	18 (72.0)	21 (84.0)	17 (68.0)
Reproductive characteristics					
Infertility diagnosis, *n* (%)					
Female factor	23 (23.0)	8 (32.0)	7 (28.0)	6 (24.0)	7 (28.0)
Ovulation disorders	10 (10.0)	5 (20.0)	2 (8.0)	3 (12.0)	2 (8.0)
DOR	3 (3.0)	0 (0.0)	3 (12.0)	1 (4.0)	0 (0.0)
Tubal	7 (7.0)	2 (8.0)	1 (4.0)	2 (8.0)	4 (8.0)
Endometriosis	2 (2.0)	0 (0.0)	1 (4.0)	0 (0.0)	1 (4.0)
Uterine	1 (1.0)	1 (4.0)	0 (0.0)	0 (0.0)	1 (4.0)
Male factor	36 (36.0)	10 (40.0)	7 (28.0)	11 (44.0)	9 (36.0)
Unexplained	41 (41.0)	7 (28.0)	11 (44.0)	8 (32.0)	9 (36.0)
Treatment protocol, *n* (%)					
Follicular phase GnRH antagonist	11 (11.0)	4 (16.0)	1 (4.0)	3 (12.0)	5 (20.0)
Follicular phase GnRH agonist	8 (8.0)	2 (8.0)	1 (4.0)	4 (16.0)	1 (4.0)
Luteal phase GnRH agonist	81 (81.0)	19 (76.0)	23 (92.0)	18 (72.0)	19 (76.0)
Day 3 FSH, IU/L	6.9 ± 2.0	7.0 ± 2.2	6.6 ± 1.9	7.0 ± 2.3	6.6 ± 1.9
Embryo transfer day, *n* (%)					
No embryos transferred	12 (12.0)	4 (16.0)	4 (16.0)	3 (12.0)	0 (0.0)
Day 2	5 (5.0)	1 (4.0)	0 (0.0)	2 (8.0)	1 (4.0)
Day 3	57 (57.0)	14 (56.0)	12 (48.0)	12 (48.0)	17 (68.0)
Day 5	26 (26.0)	6 (24.0)	9 (36.0)	8 (32.0)	7 (28.0)
Embryos transferred, *n* (%)					
No embryos transferred	12 (12.0)	4 (16.0)	4 (16.0)	3 (12.0)	0 (0.0)
1 embryo	12 (12.0)	3 (12.0)	2 (8.0)	1 (4.0)	5 (20.0)
2 embryos	60 (60.0)	13 (52.0)	16 (64.0)	16 (64.0)	17 (68.0)
≥3 embryos	16 (16.0)	5 (20.0)	3 (12.0)	5 (20.0)	3 (12.0)
Dietary characteristics					
Total energy, kcal/d	1842 ± 567	1961 ± 593	1733 ± 438	1891 ± 443	1843 ± 723
Folate, μg DFE/d	1842 ± 693	1665 ± 806	2006 ± 772	1920 ± 443	2165 ± 677*
Supplemental folate, μg/d	616 ± 342	556 ± 439	680 ± 358	662 ± 420	726 ± 317
Vitamin B-12, μg/d	39 ± 109	74 ± 163	56 ± 138	15 ± 14	76 ± 169
Multivitamin, *n* (%)	91 (91.0)	22 (88.0)	23 (92.0)	22 (88.0)	23 (92.0)
Folic acid supplement, *n* (%)	21 (21.0)	4 (16.0)	7 (28.0)	7 (28.0)	5 (20.0)

[1]*$P < 0.05$ for difference across quartiles. Differences were tested by using a Kruskal-Wallis test for continuous variables and a chi-square test for categorical variables. DFE, dietary folate equivalents; DOR, diminished ovarian reserve; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; Q, quartile.

[2]Mean ± SD (all such values).

TABLE 2
Associations between serum folate and vitamin B-12 concentrations and clinical outcomes after assisted reproduction in 100 women (154 initiated cycles) from the Environment and Reproductive Health Study (2007–2013)[1]

	Implantation		Clinical pregnancy		Live birth	
Quartile (minimum–maximum)	Cases/cycles, %	RR (95% CI)	Cases/cycles, %	RR (95% CI)	Cases/cycles, %	RR (95% CI)
Serum folate, ng/mL						
Q1 (9.7–16.5)	22/45 (48.9)	1.00 (ref)	18/45 (40.0)	1.00 (ref)	14/45 (31.1)	1.00 (ref)
Q2 (16.6–20.2)	20/35 (57.1)	1.23 (0.85, 1.77)	15/35 (42.9)	1.02 (0.62, 1.67)	10/35 (28.6)	0.77 (0.38, 1.54)
Q3 (20.3–26.3)	23/39 (58.9)	1.23 (0.90. 1.69)	22/39 (56.4)	1.35 (0.90, 2.02)	17/39 (43.6)	1.19 (0.68, 2.09)
Q4 (26.4–154.2)	22/35 (62.9)	1.31 (0.89, 1.93)	21/35 (60.0)	1.50 (0.98, 2.32)	18/35 (51.4)	1.62 (0.99, 2.65)
P-trend		0.26		0.04		0.01
Serum vitamin B-12, pg/mL						
Q1 (162–438)	25/43 (58.1)	1.00 (ref)	20/43 (46.5)	1.00 (ref)	12/43 (27.9)	1.00 (ref)
Q2 (439–534)	23/46 (50.0)	0.89 (0.62, 1.28)	20/46 (43.5)	0.92 (0.61, 1.39)	16/46 (34.8)	1.19 (0.67, 2.10)
Q3 (535–701)	15/32 (46.9)	0.82 (0.53, 1.27)	14/32 (43.8)	0.91 (0.57, 1.46)	11/32 (34.4)	1.13 (0.57, 2.24)
Q4 (702–2176)	24/33 (72.7)	1.31 (0.95, 1.80)	22/33 (66.7)	1.43 (0.98, 2.08)	20/33 (60.6)	2.04 (1.14, 3.62)
P-trend		0.03		0.01		0.008

[1]All analyses were conducted by using generalized estimating equations with binomial distribution and log link function with adjustment for age (continuous), BMI (continuous), and race (white, other). Tests for trend across quartiles were conducted by using a variable with the median serum concentration in each quartile as a continuous variable. Q, quartile; ref, reference.

When women were cross-classified into categories by serum folate and vitamin B-12 concentrations, evidence suggested an interaction (**Figure 2**); however, the overall test for interaction did not show significance (*P*-interaction = 0.58). Compared with women with both serum folate and vitamin B-12 concentrations below the median, women with both serum folate and vitamin B-12 concentrations above the median had 1.92 (95% CI: 1.12, 3.29) times the probability of live birth. This translated into an adjusted difference in live birth rates of 26% (95% CI: 10%, 0.48%) between these extreme categories ($P = 0.02$)

When we investigated early ART endpoints, serum folate was marginally associated with higher fertilization rates (*P*-trend = 0.07); however, no other associations emerged (**Table 3**). When we investigated pregnancy loss among cycles with a successful implantation ($n = 87$), the adjusted percentage of cycles lost after implantation was significantly lower in women with the highest compared with the lowest serum vitamin B-12 concentrations

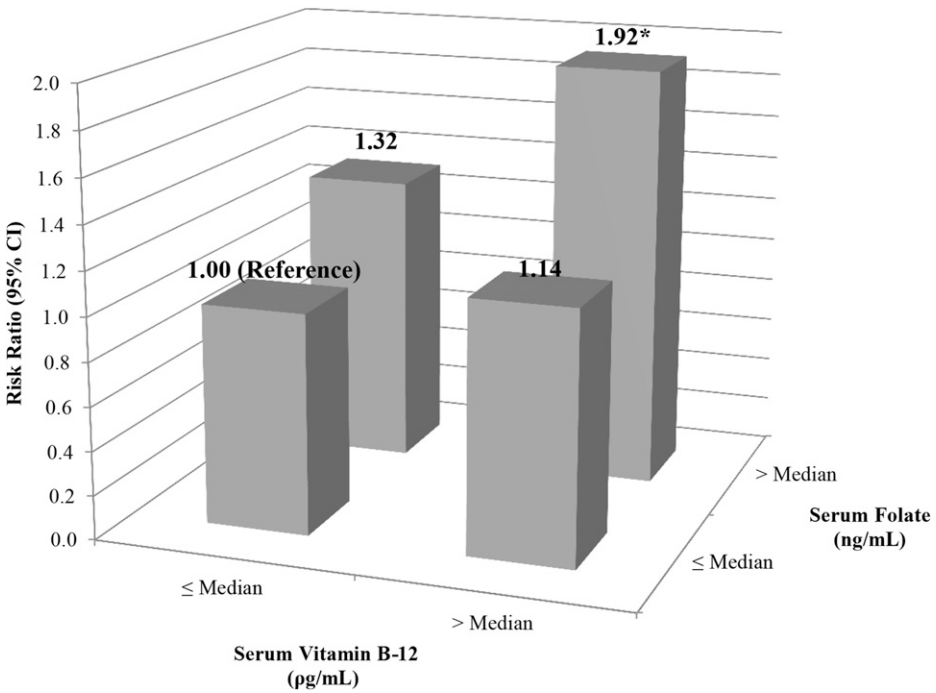

FIGURE 2 Interaction between serum folate and vitamin B-12 concentrations and live birth after assisted reproduction in 100 women (154 initiated cycles) from the Environment and Reproductive Health Study (2007–2013). The analyses were conducted by using generalized estimating equations with a binomial distribution and log link function with adjustment for age (continuous), BMI (continuous), and race (white, other). *P*-interaction = 0.58. *$P < 0.05$ compared with the reference category of women with both serum folate and vitamin B-12 concentrations below the median.

TABLE 3
Associations between serum folate and vitamin B-12 concentrations and early ART outcomes in 100 women (141 fresh IVF cycles with egg retrieval) from the Environment and Reproductive Health Study (2007–2013)[1]

	Adjusted mean (95% CI)			
Quartile (minimum–maximum)	Total oocyte yield	M2 oocytes	Fertilization rate	Proportion with ≥1 best-quality embryo
Serum folate, ng/mL				
Q1 (9.7–16.6)	10.9 (9.3, 12.8)	9.4 (8.0, 11.2)	0.59 (0.51, 0.69)	0.45 (0.32, 0.64)
Q2 (16.6–20.2)	13.0 (11.2, 15.1)	11.2 (9.6, 13.0)	0.65 (0.58, 0.72)	0.45 (0.30, 0.68)
Q3 (20.3–26.3)	10.7 (9.0, 12.7)	8.5 (7.3, 9.9)	0.69 (0.62, 0.78)	0.62 (0.46, 0.84)
Q4 (26.4–154.2)	12.4 (10.0, 15.6)	10.8 (8.7, 13.5)	0.70 (0.63, 0.78)	0.52 (0.38, 0.72)
P-trend	0.58	0.61	0.07	0.50
Serum vitamin B-12, pg/mL				
Q1 (162–438)	11.3 (9.5, 13.4)	9.7 (8.2, 11.5)	0.67 (0.60, 0.75)	0.52 (0.37, 0.74)
Q2 (439–534)	12.5 (10.4, 15.0)	10.3 (8.7, 12.3)	0.65 (0.58, 0.72)	0.47 (0.36, 0.63)
Q3 (535–701)	10.1 (8.1, 12.8)	8.7 (6.8, 11.2)	0.72 (0.66, 0.80)	0.51 (0.35, 0.74)
Q4 (702–2176)	12.1 (10.6, 13.9)	10.2 (8.8, 12.0)	0.60 (0.51, 0.72)	0.56 (0.38, 0.84)
P-trend	0.92	0.91	0.47	0.68
Low folate, low vitamin B-12	12.2 (10.6, 14.1)	10.4 (9.0, 11.9)	0.62 (0.56, 0.68)	0.52 (0.33, 0.81)
Low folate, high vitamin B-12	10.9 (9.0, 13.2)	9.8 (7.7, 12.5)	0.62 (0.50, 0.77)	0.60 (0.44, 0.80)
High folate, low vitamin B-12	11.5 (9.0, 14.7)	9.6 (7.7, 12.5)	0.72 (0.66, 0.80)	0.54 (0.38, 0.77)
High folate, high vitamin B-12	11.4 (9.6, 13.4)	9.4 (7.9, 11.2)	0.67 (0.59, 0.76)	0.42 (0.31, 0.58)

[1]All analyses were conducted by using generalized estimating equations with Poisson distribution for oocyte counts and binomial distribution for rates and proportions and the log link function for all outcomes. All models were adjusted for age (continuous), BMI (continuous), and race (white, other). Tests for trend across quartiles were conducted by using a variable with the median serum concentration in each quartile as a continuous variable. ART, assisted reproductive technology; IVF, in vitro fertilization; Q, quartile.

(18% compared with 47%; *P*-trend = 0.06). Similar trends were observed in a comparison of women with the highest compared with lowest serum folate (19% compared with 32%, *P*-trend = 0.12) and women with high folate and high vitamin B-12 compared with low folate and low vitamin B-12 (18% compared with 45%; $P = 0.05$).

The results were similar when the analyses were restricted to the first ART cycle per women, although CIs were not as precise (data not shown). Similar, albeit attenuated, results were found when dietary folate and vitamin B-12 (as estimated from the food-frequency questionnaire) were analyzed rather than the serum concentrations; the RRs for live birth were 1.05 (95% CI: 1.01, 1.10) per 200 μg DFE/d and 1.08 (95% CI: 0.93, 1.24) per 100 μg dietary vitamin B-12/d.

DISCUSSION

Our results indicate that higher serum concentrations of folate and vitamin B-12 increase the chance of live birth after ART. Moreover, women with higher concentrations of both serum folate and vitamin B-12 had the greatest likelihood of reproductive success. Analysis of intermediate endpoints suggests that folate and vitamin B-12 may exert their favorable effects on pregnancy maintenance after implantation. Serum folate also appears to be beneficial for fertilization.

The positive association between serum folate and vitamin B-12 on live birth after ART confirms our previous findings on dietary intake of these nutrients (15), but it is not entirely consistent with 3 other studies from Europe (9–11). Important differences between these studies should be noted in lieu of the incongruent findings. First, unlike our study, these studies excluded all cycles that failed before embryo transfer. The study by Haggarty et al. (10) further excluded cycles ending in implantation failure, ectopic pregnancy, termination, stillbirth, or neonatal death. If higher concentrations of folate or vitamin B-12 prevent any of these adverse outcomes from occurring, excluding them would bias the results toward the null. Second, because this study took place in European countries where the food supply is not fortified, folate concentrations were much lower than those observed in our study.

Despite these inconsistencies, considerable evidence supports a beneficial effect of folate and vitamin B-12 on outcomes of ART. Several studies from Europe have shown that folate may improve total and mature oocyte counts (6), embryo quality (7), and pregnancy rates (8) after infertility treatment. Moreover, serum and follicular fluid vitamin B-12 concentrations were positively correlated with embryo quality in a cohort of women from the Netherlands. Whereas our results on live birth were not entirely consistent with those of Haggarty et al. (10), these authors found a significantly higher rate of twin births in women with higher folate and vitamin B-12 status. Given that 91% of the women in this study had multiple embryo transfers, these results suggest that these B vitamins might increase the likelihood of embryo survival. Our findings that folate and vitamin B-12 concentrations might protect against pregnancy losses after implantation are also supported by work in pregnancies of natural conception, which have found that folic acid supplementation [and high vitamin B-12 intake (22)] is associated with a reduced risk of spontaneous abortion (22–24).

The suggestion of an interaction between folate and vitamin B-12 on outcomes of ART has not been previous evaluated; however, biological rationale supports this finding. Vitamin B-12 is a cofactor for folate-dependent methionine synthase, which is involved in homocysteine remethylation (25). The methionine derivative *S*-adenosylmethionine is the most important methyl donor in the body for the methylation of lipids, proteins, and DNA. A deficiency in *S*-adenosylmethionine reduces DNA methylation and consequently leads to hypomethylation of DNA, which may lead to aberrant patterns of gene expression

(26). Synthesis, repair, and methylation of DNA are crucial in gametogenesis, fertilization, and pregnancy (27, 28). Another consequence of impaired methionine synthase is the accumulation of homocysteine, which may induce cytotoxic and oxidative stress and lead to impaired oocyte maturation, embryo development, and endothelial cells (29, 30). Exposure of trophoblast cells to elevated homocysteine may also increase cellular apoptosis and lead to inhibition of trophoblastic function, which is essential for successful placentation (31). Nevertheless, whereas live birth rates were highest among women with high serum folate and vitamin B-12 concentrations, the interaction was not statistically significant and the study was underpowered to identify interactions.

The limitations and strengths of this study are worth noting. First, whereas serum concentrations of folate and vitamin B-12 are objectives measure of dietary intake over the past 3 mo, they are not measured without error. Because of the prospective nature of our study and the measurement of biomarkers without regard to case status, however, any errors are expected to be nondifferential with respect to our outcomes. Second, whereas we assessed confounding by a variety of demographic, dietary, and reproductive characteristics, the possibility of residual confounding exists because of the observational nature of this study. The generalizability of our study to women presenting at infertility clinics worldwide is unclear because our women have much higher serum folate concentrations (median = 20.5 ng/mL) than comparable populations in Europe (7) (median = 13.5 ng/mL) because of our fortified food supply and high use of supplements (32, 33). Serum folate concentrations in our population were also much higher than those measured in women in NHANES (12.7 ng/mL) (34). Serum concentrations of vitamin B-12, however, were more comparable with those in European infertility populations (535 pg/mL compared with 430 pg/mL) and in women in the United States based on NHANES (468 pg/mL) (7, 34). In addition, even though the assays used in our study are approved for clinical use, folate electrochemiluminescence assays are known to systematically differ from microbiologic folate assays such that the association identified in our study may be biased toward the null (35). Despite these limitations, our study was strengthened by the use of a prospective design and the ability to evaluate early endpoints that cannot be observed in couples attempting to conceive naturally. In addition, by analyzing the data from all women who initiated treatment, and not just from only women who underwent an embryo transfer, we were able to avoid the potential of bias introduced by conditioning on an important intermediate endpoint (36). Whereas none of the women in our analysis were folate deficient and very few were vitamin B-12 deficient, we still benefitted from having a wide range of folate and vitamin B-12 concentrations in our population, which increased our power to discern significant associations.

In conclusion, we found that high concentrations of folate and vitamin B-12 in serum are associated with an increased chance of live birth after ART. These findings support the importance of preconception folic acid supplementation and suggest the additional intake of vitamin B-12. Given that live birth rates per initiated ART cycle have plateaued for approximately a decade in the United States, a randomized trial of high-dose supplementation with folic acid and vitamin B-12 before planned ART warrants serious consideration.

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