GYNECOLOGY

Preconception folate status and reproductive outcomes among a prospective cohort of folate-replete women

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BACKGROUND: Most studies of folate metabolism and reproduction have been conducted after pregnancy and in folate-deficient populations. However, measurement of maternal folate status preconceptionally may be most relevant to certain folate-linked early processes preceding a successful pregnancy, and there has been a major increase in folate concentrations in women of childbearing age in high resource settings.

OBJECTIVE: To examine associations between preconceptional biomarkers of maternal folate status (folate and homocysteine) and reproductive outcomes in folate-replete women.

STUDY DESIGN: Cohort nested within the Effects of Aspirin in Gestation and Reproduction trial, a block-randomized, double-blind, placebo-controlled trial whereby women were randomized to daily low-dose aspirin (81 mg/day) or placebo and all women received folic acid (400 μg/day). In total, 1228 women with 1−2 previous pregnancy losses and no documented infertility were recruited from 4 clinical sites in the United States (2006−2012) and were attempting pregnancy for up to 6 menstrual cycles. Log-binomial regression models were used to estimate relative risks and 95% confidence intervals between preconception serum folate and plasma homocysteine for anovulation, pregnancy, and pregnancy loss.

RESULTS: Greater plasma homocysteine was nonlinearly associated with greater risks of pregnancy loss only among women with 2 previous losses: a relative risk of 1.43 (95% confidence interval, 1.08−1.89) was found for plasma homocysteine concentrations at the study median of 8.0 μmol/L compared with a US population median of 6.0 μmol/L. No meaningful relationships were found between serum folate and any reproductive outcome or between plasma homocysteine and anovulation or becoming pregnant.

CONCLUSION: These data justify further study of the role of folate and homocysteine metabolism in normal and abnormal early pregnancy.

Key words: folic acid, homocysteine, miscarriage, pregnancy loss

Insufficient folate status has been associated with many reproductive complications, including anovulation,[1] subfertility,[2] and early pregnancy loss.[1] Homocysteine is commonly used as an indicator of folate status. An amino acid involved in one-carbon metabolism of folate, greater homocysteine levels have been associated with hormonal changes across the menstrual cycle.[1] It is hypothesized that elevated homocysteine may be related to vascular changes associated with abnormal placentation,[3−6] which may increase the risk of pregnancy loss.

Measurement of maternal folate status preconceptionally may be most relevant to certain folate-linked early processes preceding a successful pregnancy, such as ovulation, or implantation whereby the placenta begins to differentiate. However, most studies of folate metabolism and reproduction have been conducted either during or after pregnancy,[5,7−11] subsequent to placental differentiation and early pregnancy losses. Although historical studies on this topic have been conducted in relatively folate-deficient populations, examination of folate-replete women would yield findings more generalizable to the US population of women actively attempting natural conception. We therefore investigated how biomarkers of maternal folate status, the result of B vitamin intake and folate metabolism, are prospectively related to reproductive outcomes among folate-replete women with a history of pregnancy loss.

Subjects and Methods

Study sample

Data were drawn from the Effects of Aspirin in Gestation and Reproduction (EAGeR) trial, a block-randomized, double-blind, placebo-controlled trial designed to study the effects of preconception low-dose aspirin on live birth in women with 1−2 previous pregnancy losses.[12] Study design, methods, inclusion, and exclusion criteria have been described previously.[12,13] Participant flowchart is provided in Figure 1. To summarize, 1228 women 18−40 years of age with no known major medical conditions or documented infertility were recruited from 4 clinical sites in the United States (2006−2012). Inclusion was limited to women having regular menstrual cycles and attempting to conceive naturally. Women were randomized to daily low-dose aspirin (81 mg/day) or placebo and all women received folic acid (400 μg/day). Women were followed up to 6 menstrual cycles and through pregnancy for those who became pregnant; study treatment continued through week 36 of pregnancy. The date of first patient's enrollment was June 15, 2007. The trial was registered on ClinicalTrials.gov (#NCT00467363).

Human and nonhuman experimentation

The institutional review board at each study site and data coordinating center

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AJOG at a Glance

Why was the study conducted?

Most studies of folate metabolism and reproduction have been conducted in folate deficient populations and among nonpregnant women.

Key findings

Our findings suggest that preconception homocysteine, but not folate, is linked to pregnancy loss among folate-replete women with a history of 2 pregnancy losses.

What does this add to what is known?

These data justify further study of the role of folate and homocysteine metabolism in normal and abnormal early pregnancy.

approved the trial protocol and obtained approvals: University of Utah (1002521), date May 24, 2007; Scranton, Pennsylvania (HHSN275200403394) date May 2007; University at Buffalo (SPM0900107A), date December 20, 2007; and University of Colorado (08-0982), date April 2009. Trial registration date: April 27, 2007. All participants provided written informed consent before enrolling.

Serum folate and plasma homocysteine measurement

Serum folate and plasma homocysteine levels were measured at baseline, before consumption of study medication. Preconception serum sample collection coincided with days 2−4 of each participant's menstrual cycle and stored at −80°C until assay. Serum folate was measured by the Folate III reagent/competitive immunoassay method/electrochemiluminescence using the Roche COBAS 6000 chemistry analyzer (Roche Diagnostics, Indianapolis, IN). The interassay laboratory coefficients of variation were 9.9% and 6.3% at 8.63 nmol/L and 26.73 nmol/L, respectively. Total plasma homocysteine was measured by liquid chromatography/tandem mass spectrometry using Waters 2795 Liquid Chromatography (Waters Corp, Milford, MA) equipped with an autosampler and column oven. Data were obtained using the MassLynx software (Waters Corp) with automated data processing by QuanLynx software (Waters Corp). The interassay laboratory coefficients of variation are 4.6 and 6.8% at mean concentrations of 1.0 and 22.6 μmol/L, respectively.

Outcome ascertainment

Reproductive outcomes included anovulation, pregnancy, and pregnancy loss.[13−15]

Anovulation

Fertility monitors used for intercourse timing (Clearblue Easy Fertility Monitor; Alere, Waltham, MA) measured luteinizing hormone (LH) and estrone-3-gluconuride concentrations in first-morning urine.[16] The test stick measures urinary LH by a classical sandwich assay, and the monitor optically reads the intensity of the line. Hormone values were downloaded from the internal memory chip at the end of each cycle.

Ovulation was detected using fertility monitors for up to 6 cycles of preconception follow-up, and urinary luteal pregnanediol-3-glucuronide (a progesterone metabolite) measurements were used to improve the sensitivity of ovulation detection in the first 2 cycles of study participation. Cycles without a peak reading on the monitor or daily LH concentrations <2.5 times the average of the previous 5 days were considered anovulatory, as well as cycles with pregnanediol-3-glucuronide <5 μg/mL.[16−18] For fertility monitor criteria to apply, data must have been available on the 15th day before the end of the cycle to classify a lack of peak, and for the moving average method, tests on at least 2 of the previous 5 days with at least 1 in the immediate 2 previous days for a valid average were required; otherwise, the cycle was unable to be categorized by the fertility monitor criteria. Cycles with insufficient data for all methods were classified as missing (n = 397). Reasons for missing anovulation data were infrequent fertility monitor testing (≤10 testing days), missing tests on or around the anticipated day of LH surge (15 days before the start of menses[19]), non-use of the fertility monitor, or testing malfunctions. Cycles resulting in pregnancy were considered ovulatory.

Pregnancy

Pregnancies were identified by human chorionic gonadotropin (hCG) in 3 ways: (1) a positive urine pregnancy test using a home pregnancy kit (Quidel Quickvue One step hCG test; Quidel Corporation, San Diego, CA), (2) an in-clinic urine test among women reporting a missed menses, or (3) by free beta hCG measured in spot urine samples from visits occurring between cycle days 2 and 4, and in daily first-morning urine samples from the last 10 cycle-days of participants' first 2 menstrual cycles (initial test: catalogue no. RIS0011R, BioVendor, Asheville, NC; confirmatory test: catalogue no. 4221-16, Diagnostic Automation Inc, Calabasas, CA). Following a positive urine pregnancy test, ultrasound-confirmed pregnancy was a secondary outcome defined per study protocol by the presence of a gestational sac via ultrasound at approximately 6.5 weeks of gestation.

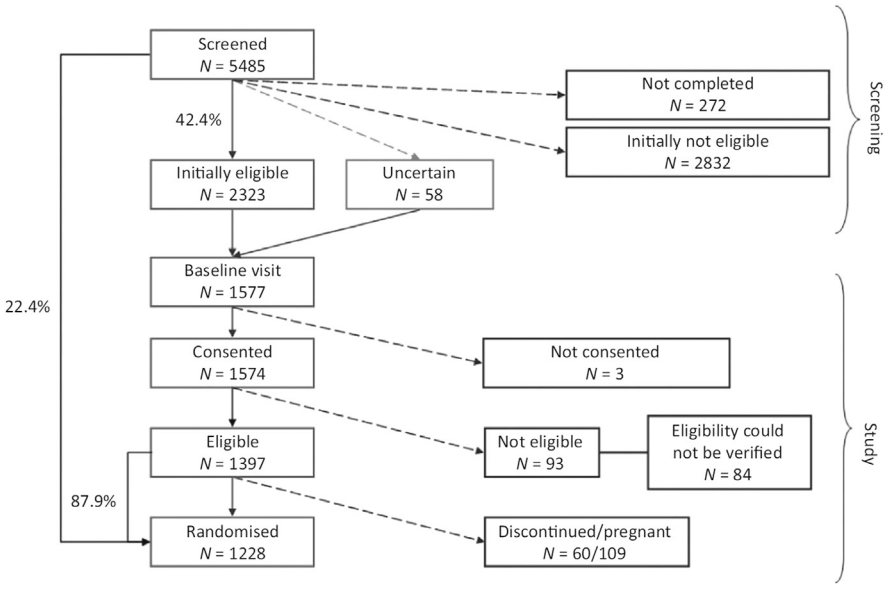
Pregnancy loss

Pregnancy loss was identified by the absence of clinical signs of pregnancy following a positive urine pregnancy test or positive free beta hCG in stored urine samples, or by the participant or clinician after ultrasound confirmation.

Covariates

At baseline, participants completed questionnaires on demographics, lifestyle habits, socioeconomic status, and reproductive history. In the baseline

FIGURE 1
Effects of aspirin in gestation and reproduction recruitment stages



Number of women who were screened, eligible, and completed baseline and randomization visits. Reproduced with permission from Schisterman et al.[12]

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questionnaire, women were asked whether they were currently taking a supplement containing folic acid, but dose was not assessed. Height and weight were measured at baseline to calculate body mass index (BMI; kg/m^2), which was not normally distributed. As the reciprocal of BMI more closely approximated normality, this transformation was used to account for BMI in adjusted models.

Statistical analyses

Serum folate, approximately normally distributed, was summarized as means and standard deviations with *t* tests or analysis of variance by categorical baseline characteristics. Plasma homocysteine, approximately log-normally distributed, was summarized as medians and interquartile ranges with Wilcoxon Mann−Whitney tests by categorical baseline characteristics. Relationships between exposures and continuous maternal characteristics were examined using linear regression (homocysteine was log-transformed) and restricted cubic splines to examine potential nonlinearities.

Multiple imputation was used to address missing data for women with missing folate (n = 195; 15.9%), homocysteine (n = 22; 1.8%), BMI (n = 20; 1.6%), and anovulation (n = 397; 13% of cycles). Chained equations with all indicated covariates generated 50 datasets to analyze pregnancy, pregnancy loss, and live birth. Twenty datasets were separately generated to analyze anovulation due to multiple cycles per woman.[20]

Given that pregnancy loss is conditional on becoming pregnant, these analyses were conducted only among women who became pregnant, with inverse probability weighs used to account for the potential that women who became pregnant may have had different characteristics than those who did not. Similarly, as participants who withdrew also may have differed than those who completed the study, inverse probability weights were separately constructed for study withdrawals. Pregnancy and withdrawal weights were based on factors associated with becoming pregnant and study withdrawal, respectively. Analytic details on calculation of weights are included in the Supplemental Materials and Methods.

Log-binomial regression models were used to estimate relative risks (RRs) and 95% confidence intervals between serum folate and plasma homocysteine for anovulation, pregnancy, and pregnancy loss. An ad hoc analysis examined time to pregnancy. Estimates were calculated for an increase of 15 nmol/L serum folate or 2 μmol/L plasma homocysteine, each approximately 1 standard deviation of exposure. Ad hoc analyses for homocysteine were stratified by previous loss number. Unadjusted models and models adjusted for age, reciprocal of BMI, previous number of pregnancy losses loss (1, 2), and parity (0, 1+) were examined. Because there were no relationships between loss and marital status, race, smoking, education, income, or alcohol intake in this population, these covariates were not included in adjusted models. Restricted cubic splines were used to examine potential nonlinearities (Supplemental Materials and Methods). Linear models used multiple imputation and weighting. Splines were weighted and utilized unimputed data. Analyses were conducted using SAS, version 9.4 (SAS Institute Inc., Cary, NC).

Results

Study sample characteristics

Among 1228 study participants, there were 797 (64.9%) pregnancies and 188 (23.6%) pregnancy losses. One hundred sixty-nine of 188 (90%) of pregnancy losses occurred within 12 weeks of gestation. There were 1222 women who contributed 4266 total cycles; 6 women withdrew from the study before contributing any cycles. Among cycles with sufficient data (n = 3869), 486 (12.6%) were classified as anovulatory. Three hundred thirty-five of 1228 (27.3%) women were classified as having at least one anovulatory cycle.

Approximately 80% of women reported taking a supplement containing folic acid at baseline (Table 1). Serum folate measurements ranged from 14.5 to 90.1 nmol/L (median: 58.2 nmol/L). Plasma homocysteine ranged from 2.9 to 31.6 μmol/L

TABLE 1
Preconception folate and homocysteine by covariates

Covariate		Folate,[a] nmol/L		Log homocysteine,[a] μmol/L
Continuous	n	β (SE)	n	β (SE)
Age	1033	0.16 (0.098)	1206	0.0045 (0.0013)
BMI	1015	−0.52 (0.069)	1186	0.0016 (0.00093)
Missing	20		20	
		Folate,[b] nmol/L		Homocysteine,[c] μmol/L
Categorical	n	mean (SD)	n	median (IQR)
Marital status				
Married	935	57.8 (14.6)	1106	7.9 (7.0–9.1)
Not married	98	45.8 (14.7)	100	8.4 (7.3–9.9)
Parity				
0	480	57.7 (14.0)	564	8.1 (7.1–9.3)
1+	553	55.8 (15.7)	642	7.8 (6.9–9.0)
Loss number				
1	681	57.2 (14.8)	807	7.9 (7.0–9.1)
2	352	55.6 (15.3)	399	8.0 (6.9–9.3)
Race				
White	973	57.2 (14.7)	1142	7.9 (6.9–9.1)
Nonwhite	60	47.0 (16.5)	64	8.4 (7.7–9.5)
Education				
Up to high school	152	51.5 (14.7)	163	8.1 (7.0–9.4)
Beyond high school	880	57.6 (14.9)	1042	7.9 (7.0–9.1)
Missing	1	34.3 (n/a)	1	8.2 (n/a)
Annual income (USD)				
≤19,999	85	52.0 (14.9)	92	7.9 (6.9–9.1)
20–39,999	280	55.9 (15.5)	308	8.0 (7.0–9.1)
40–74,999	145	59.3 (13.9)	178	8.0 (7.0–9.1)
75–99,999	114	59.0 (15.0)	148	8.0 (7.2–9.5)
≥100,000	408	56.5 (14.8)	479	7.9 (6.9–9.2)
Missing	1	54.2 (n/a)	1	7.4 (n/a)
Smoking status				
Any	135	51.8 (15.1)	147	8.5 (7.3–9.9)
None	888	58.0 (14.6)	1049	7.9 (6.9–9.1)
Missing	10	53.3 (13.1)	10	8.3 (8.0–9.2)

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(median: 8.0 μmol/L). Preconception serum folate and log plasma homocysteine were positively associated with maternal age (Table 1). Serum folate was negatively, and plasma homocysteine was positively, associated with BMI. Greater mean folate values were observed among women who were married, white, had beyond a high school education, had greater incomes, and did not smoke or drink alcohol, whereas greater median homocysteine values were observed among women who were not married, nonwhite, and who reported smoking daily and drinking in the previous year.

TABLE 1
Preconception folate and homocysteine by covariates *(continued)*

Categorical	n	Folate,[b] nmol/L mean (SD)	n	Homocysteine,[c] μmol/L median (IQR)
Alcohol consumption (past year)				
Any	336	52.8 (15.4)	398	8.2 (7.1–9.5)
None	683	58.6 (14.6)	793	7.8 (6.9–9.0)
Missing	14	54.7 (7.7)	15	8.1 (7.5–8.4)
Taking folic acid (baseline)				
Yes	816	56.6 (14.9)	957	8.0 (7.0–9.2)
No	202	57.4 (15.3)	234	7.8 (7.0–9.0)
Missing	15	50.2 (14.1)	15	8.1 (6.9–9.1)

ANOVA, analysis of variance; *BMI*, body mass index; *IQR*, interquartile range; *n/a*, not available; *SD*, standard deviation; *SE*, standard error; *USD*, US dollars.

[a] Linear regression; linearity confirmed through restricted cubic splines; homocysteine log transformed to attain approximate normality; [b] *t* test and ANOVA for approximate normal distribution of serum folate; [c] Wilcoxon Mann–Whitney test for non-normal distribution of plasma homocysteine.

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Folate, homocysteine, and reproductive outcomes

There was no evidence of meaningful relationships between preconception serum folate and any outcome or between preconception plasma homocysteine and anovulation, pregnancy, or time to pregnancy in linear models (Table 2, Table 3, Supplemental Table 1); restricted cubic splines did not indicate meaningful deviations from linearity.

Plasma homocysteine was associated with a greater risk of pregnancy loss in a linear model (RR, 1.12 [1.00–1.23] per 2 μmol/L increase). When stratified by previous loss number, this association was only evident among women with 2 previous losses. The RR for each 2 μmol/L increase in plasma homocysteine was 1.15 (1.00–1.29) for 2 previous losses, compared with 1.03 (0.84–1.23) for 1 previous loss. Restricted cubic splines indicated deviation from linearity only among women with two prior losses (Figure 2). Compared with 6.0 μmol/L (congruent with National Health and Nutrition Examination Survey US population estimates[21]), at 8.0 μmol/L (the study population median), the unadjusted nonlinear model for pregnancy loss produced a RR of 1.43 (1.08–1.89), compared with 1.11 (0.98–1.25) for the unadjusted linear model.

Comment

Principal findings

In this prospective cohort of healthy folate-replete women attempting pregnancy after 1–2 previous pregnancy losses, serum folate was not associated with anovulation, pregnancy, or pregnancy loss. Although plasma homocysteine was not associated with anovulation or becoming pregnant, greater preconception plasma homocysteine was associated with a greater risk of pregnancy loss only among women with 2 previous losses.

Strengths and limitations of study

This is the first study to examine preconceptional biomarkers of folate status and reproductive outcomes among healthy folate-replete women. Measuring circulating folate biomarkers accounts for genetic variants that affect one-carbon metabolism. Measurement on days 2–4 of each woman's cycle likely

TABLE 2
Weighted[a] and pooled[b] RRs and 95% CIs between preconception serum folate[c] and anovulation, pregnancy, and pregnancy loss

Outcome by exposure	Unadjusted RR (95% CI)	Fully adjusted RR (95% CI)
Serum folate		
Anovulation	0.91 (0.80–1.02)	0.98 (0.86–1.10)
Pregnancy	1.01 (0.98–1.05)	1.00 (0.98–1.03)
Pregnancy loss	1.06 (0.90–1.21)	1.07 (0.91–1.22)

CI, confidence interval; *RR*, relative risk.

[a] Anovulation models (n = 4266 cycles, 1222 women) weighted by the inverse of the cluster size; pregnancy models (n = 1100) weighted for withdrawal before pregnancy (n = 128; 10.4%); pregnancy loss models (n = 657) weighted for weighted for withdrawal (n = 140; 11.4% women) and non-pregnancy (n = 431, 35.1% women); [b] Estimates individually calculated for each of 20 imputations for anovulation and 50 imputations for pregnancy and pregnancy loss; imputation-specific estimates pooled into a single summary measure estimate for each outcome; [c] Calculated for an increase of 15 nmol/L serum folate, approximately 1 standard deviation.

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TABLE 3
Weighted[a] and pooled[b] relative risks and 95% confidence intervals between preconception plasma homocysteine[c] and pregnancy loss

Outcome by exposure	1 or 2 previous losses		1 previous loss		2 previous losses	
	Unadjusted	Fully adjusted	Unadjusted	Fully adjusted	Unadjusted	Fully adjusted
Plasma homocysteine	RR (95% CI)	RR (95% CI)	RR (95% CI)	RR (95% CI)	RR (95% CI)	RR (95% CI)
Anovulation	1.01 (0.90–1.11)	1.00 (0.89–1.11)	1.02 (0.86–1.18)	0.99 (0.84–1.15)	0.99 (0.86–1.12)	0.99 (0.84–1.13)
Pregnancy	0.95 (0.91–1.00)[d]	0.99 (0.96–1.02)	0.93 (0.87–0.99)	0.98 (0.92–1.03)	0.99 (0.92–1.05)	0.99 (0.94–1.04)
Pregnancy loss	1.12 (1.01–1.24)[d]	1.12 (1.00–1.23)[d]	1.05 (0.86–1.25)	1.03 (0.84–1.23)	1.11 (0.98–1.25)	1.15 (1.00–1.29)[d]

CI, confidence interval; *RR*, relative risk.

[a] Anovulation models (n = 4266 cycles, 1222 women) weighted by the inverse of the cluster size; pregnancy models (n = 1100) weighted for withdrawal before pregnancy (n = 128; 10.4%); pregnancy loss models (n = 657) weighted for weighted for withdrawal (n = 140; 11.4% women) and nonpregnancy (n = 431, 35.1% women); [b] Estimates individually calculated for each of 20 imputations for anovulation and 50 imputations for pregnancy and pregnancy loss; imputation-specific estimates pooled into a single summary measure estimate for each outcome; [c] Calculated for an increase of 2 μmol/L plasma homocysteine, approximately 1 standard deviation; [d] Statistically significant.

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reduced biomarker variability because fluctuations in one-carbon metabolism are influenced by changes in cyclical hormones.[22–24] Preconception measurement of folate biomarkers overcomes limitations of previous work conducted in pregnant[5] and nonpregnant[6–11,25] women, which systematically missed early losses.

Analytic methods were used to reduce the potential impact of bias. To use all study observations and improve efficiency of linear models, multiple imputation was used primarily to account for missing folate and anovulation data. Although rarely realized in practice, complete-case analysis assumes that the missing data are not related to missing or observed data. This assumption is relaxed in multiple imputation, which assumes that missing data can be related to observed data. Inverse probability weighting accounted for potential selection bias incurred by restricting pregnancy and loss analyses to women who did not withdraw and pregnancy loss analyses to women who became pregnant. In contrast to previous studies, the exposure in the current study precedes pregnancy, so pregnancy is an intermediate variable on the path to pregnancy loss. Because there may be factors associated with becoming pregnant that also are associated with pregnancy loss after becoming pregnant, unless these factors are taken into account through inverse probability weighting, selecting on the intermediate variable (eg, pregnancy) in pregnancy loss analyses can induce bias through attenuation of calculated risks.[26]

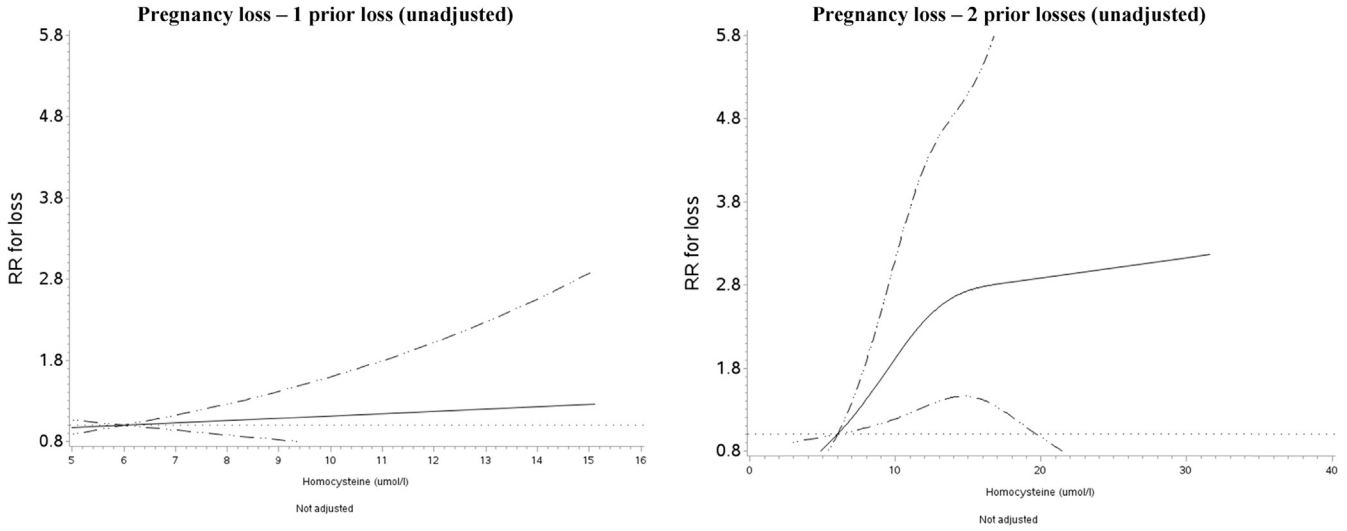
The study also has several limitations. We are unable to discern baseline folic acid supplement formulation, dosage, duration, or frequency, which was unrelated to baseline serum folate or plasma homocysteine measurements. It is possible that serum folate is not a sufficiently sensitive marker of chronic maternal folate status as compared with red blood cell folate, as serum folate is sensitive to recent ingestion of folic acid or folate-rich food.[27] Because homocysteine is less volatile, it is a better measure of long-term folate status. Given the high serum folate concentrations observed in this study, it is unlikely that this alone explains the discordant findings for folate and homocysteine. Homocysteine is influenced by B vitamins other than folate (vitamins B_{12}, B_6, and B_2) and by the *MTHFR* 677C→T variant, which were not measured in this study. Study generalizability is limited to women actively attempting to conceive following 1–2 previous losses and predominantly to white women of greater socioeconomic status living in the United States.

Comparison with other studies

This is the first study to examine preconceptional folate and homocysteine in relation to multiple reproductive outcomes in a folate-replete population of women attempting pregnancy. Our finding that greater preconception plasma homocysteine was associated with greater risks of pregnancy loss among folate-replete women with 2 previous losses is concordant with previous cross-sectional and retrospective case–control studies of recurrent pregnancy loss.[7–11,25] However, these previous studies were conducted among more folate-deplete populations of nonpregnant women. Although a more recent study of pregnant women unselected by loss history also supports these findings,[5] none of the previous studies were conducted preconception, a critical exposure window.

Although a previous study of healthy women with regular menses found that greater homocysteine was associated with an elevated risk of anovulation at the time of expected ovulation, this association was attenuated by approximately 50% during the mid-follicular phase[1] (a more comparable time point with the present study). Indeed, the hormonal milieu at different phases of the menstrual cycle may influence the observed relationships. Similar to our findings, neither folate measured at expected ovulation nor in the mid-follicular phase was associated with anovulation risk in the former study. Interestingly, the population examined in the former study was also folate-replete, so the

FIGURE 2
Restricted cubic splines for preconception plasma homocysteine and pregnancy loss



Plasma hcy (μmol/L)	RR (95% CI)
6.0	*Reference*
8.0	1.05 (0.88, 1.26)
10.0	1.11 (0.77, 1.60)
12.0	1.17 (0.67, 2.02)

Plasma hcy (μmol/L)	RR (95% CI)
6.0	*Reference*
8.0	1.43 (1.08, 1.89)
10.0	1.92 (1.19, 3.12)
12.0	2.37 (1.33, 4.24)

Anovulation models weighted for missing anovulation (n = 403; 9.4% cycles) and homocysteine (n = 22; 1.8% women); pregnancy models weighted for withdrawal before pregnancy (n = 128; 10.4%) and missing homocysteine, pregnancy loss models weighted for withdrawal (n = 140; 11.4% women), nonpregnancy (n = 431, 35.1% women), and missing homocysteine. Unadjusted relative risks defined relative to median values for homocysteine (6.0 μmol/L)[12,13] among U.S. reproductive-aged women.

BMI, body mass index; *CI*, confidence interval; *RR*, relative risk.

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pattern of identifying relationships with homocysteine, but not for folate, further support that homocysteine may be associated with adverse reproductive outcomes by mechanisms independent of folate.

Notably, there was an absence of an association between levels of serum folate and any of the reproductive outcomes evaluated in the current study. Previous studies[11,28] that reported associations between low serum folate and early pregnancy loss in women with a previous history of pregnancy loss were conducted in non−folate-replete populations. In contrast, the mean serum folate in the current study was 56.6 nmol/L, and no individuals had a negative folate balance (<7 nmol/L) or subclinical deficiency (<10 nmol/L).[27]

Meaning of the study

High homocysteine is a risk factor for vascular disease, which acts on blood vessel walls to elicit changes to the endothelium.[29] This could cause premature damage[30,31] to decidual or chorionic vasculature,[32,33] which could disrupt placentation.[34] Abnormal placental vasculature has been noted among women with elevated homocysteine levels measured early in pregnancy[5] and following pregnancy loss.[6] Among women with at least 2 consecutive early pregnancy losses, elevated total homocysteine was associated with smaller median areas and perimeters of chorionic villous vasculature, and fasting homocysteine was negatively correlated with vascular element perimeter.[6] Defective placentation has been observed in about two-thirds of pregnancy losses occurring in the first trimester.[35] Plasma homocysteine could also indirectly contribute to pregnancy loss. For instance, because greater plasma homocysteine was associated with lower luteal phase progesterone in a recent study of healthy regularly menstruating women,[1] this could contribute to luteal phase deficiency whereby the corpus luteum does not produce sufficient progesterone to maintain the early embryo.

High homocysteine is a marker for abnormal folate metabolism and/or deficiencies in B vitamins (eg, active folate, B_{12}, or B_6)[5], which can be the result of diminished homocysteine re-methylation. Given that women in the current study were folate-replete and folate was not inversely associated with adverse outcomes in this study, high homocysteine in this population may have been the result of deficiency in enzymes involved in re-methylation, such as 5,10-methylenetetrahydrofolate reductase (MTHFR).[7] In particular, a mutation in *MTHFR677* has been previously been associated with elevated

risk of pregnancy loss in some, but not all, studies.[36,37]

Unanswered questions and proposals for future research

This is the first study to examine preconceptional biological indicators of folate status and multiple reproductive outcomes among healthy folate-replete women. In this population, greater preconception plasma homocysteine, but not folate, was associated with greater risk of pregnancy loss among women with 2 previous losses. These findings provide valuable new evidence on the relationships between markers of one-carbon metabolism and pregnancy loss risk in folate-replete women attempting to conceive after a history of pregnancy loss. These data justify further evaluation to clarify the role of folate and homocysteine metabolism in normal and abnormal reproduction and early pregnancy. Because safe treatment options are available, this pathway is an attractive therapeutic target. ■

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Supplemental Methods

Multiple imputation

Chained equations with all indicated covariates generated 50 datasets to analyze pregnancy, pregnancy loss, and live birth. Twenty datasets were separately generated to analyze anovulation due to having repeated observations per woman.[1] Weighted estimates were individually calculated for each imputation and pooled into a single summary measure estimate for each outcome using SAS PROC MIANALYZE.

Calculation of weights

In anovulation models, study participants contributed menstrual cycles at risk of anovulation from the cycle of randomization until the first of the following events: human chorionic gonadotropin–detected pregnancy (the cycle resulting in pregnancy was included), completion of 6 menstrual cycles without pregnancy, or study withdrawal. To account for multiple observations per participant and informative cluster size, anovulation models were clustered by participant with an autoregressive correlation matrix[2] and weighted by the inverse of the cluster size,[3] respectively.

Inverse probability weighting accounted for potential selection bias for pregnancy and pregnancy loss, with separate weights constructed for each imputation. Pregnancy was coded as missing for participants who withdrew from the study without becoming pregnant (n = 128; 10.4%). Pregnancy loss was coded as missing for participants who did not become pregnant (n = 431; 35.0%) or who withdrew after becoming pregnant (n = 12; 1.0%).

Inverse probability weights were constructed separately for withdrawal and pregnancy status and were based on factors associated with withdrawal and/or pregnancy. Combinations of factors were selected to attain means of approximately one, a necessary condition of correct model specification.[4] Folate or log homocysteine exposure were included in each the numerator and denominator. In pregnancy models, a single weight was utilized to account for withdrawals without pregnancy. In pregnancy loss models, withdrawal and pregnancy weights were multiplied to create a combined weight.

Restricted cubic splines

Restricted cubic splines were constructed using SAS PROC GENMOD within the %GLMCURV9 macro[5] on unimputed data. For exposure-outcome models, splines used weighted log-binomial regression models. Missing folate and homocysteine exposure data were addressed using an additional inverse probability weight that was multiplied to existing weights in each analysis.

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SUPPLEMENTAL TABLE 1
Weighted[a] and pooled[b] fecundability ORs and 95% CIs between preconception serum folate[c] and plasma homocysteine[d]

Outcome by exposure	Unadjusted FOR (95% CI)	Fully adjusted[e] FOR (95% CI)
Serum folate	1.00 (0.90–1.11)	0.98 (0.88–1.09)
Plasma homocysteine	0.97 (0.86–1.08)	0.99 (0.89–1.10)
1 previous loss	0.96 (0.81–1.11)	1.00 (0.84–1.15)
2 previous losses	0.98 (0.83–1.14)	0.99 (0.84–1.14)

BMI, body mass index; *CI*, confidence interval; *FOR*, fecundability odds ratio; *OR*, odds ratio.

[a] Pregnancy models (n = 1100) weighted for withdrawal before pregnancy (n = 128; 10.4%); [b] Estimates individually calculated for each of 50 imputations; imputation-specific estimates pooled into a single summary measure estimate; [c] Calculated for an increase of 15 nmol/L serum folate, approximately 1 standard deviation; [d] Calculated for an increase of 2 μmol/L plasma homocysteine, approximately 1 standard deviation; [e] Adjusted for reciprocal of BMI, age, parity (0, 1+), and loss number (1, 2; unstratified analyses only).

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