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ORIGINAL ARTICLE ***Reproductive epidemiology***

Folate, homocysteine and the ovarian cycle among healthy regularly menstruating women

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STUDY QUESTION: How are concentrations of plasma homocysteine and serum folate associated with reproductive hormones and anovulation in regularly menstruating women?

SUMMARY ANSWER: Higher homocysteine was associated with sporadic anovulation and hormonal changes that may be indicative of impaired ovulatory function, but higher serum folate was associated only with higher luteal phase progesterone.

WHAT IS KNOWN ALREADY: Higher folate levels as well as some variants in genes relevant to one-carbon metabolism, are associated with improved reproductive outcomes and responses to fertility treatment, but only a few small studies have explored the relationship between markers of one-carbon metabolism and menstrual cycle characteristics.

STUDY DESIGN, SIZE, DURATION: The BioCycle Study (2005–2007) is a prospective, longitudinal cohort of 259 regularly menstruating women not using hormonal contraceptives or dietary supplements who were followed for up to two menstrual cycles.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Serum folate and reproductive hormones were measured up to eight times per cycle and plasma homocysteine up to three times. Linear mixed models were used to estimate associations between serum folate or plasma homocysteine and log-transformed reproductive hormone levels while accounting for multiple observations and cycles per woman. Generalized estimating equations were used to examine risk of sporadic anovulation. All models were adjusted for age, race, body mass index, cigarette and alcohol use, and energy and fiber intake.

MAIN RESULTS AND THE ROLE OF CHANCE: Higher plasma homocysteine concentrations were associated with lower total estradiol across the cycle (adjusted percent change per unit increase in homocysteine [aPC] −2.3%, 95% CI: −4.2, −0.03), higher follicle stimulating hormone around the time of expected ovulation (aPC 2.4%, 95% CI: 0.2, 4.7) and lower luteal phase progesterone (aPC −6.5%, 95% CI: −11.1, −1.8). Higher serum folate concentrations were associated with higher luteal phase progesterone (aPC per unit increase in folate 1.0%, 95% CI: 0.4, 1.6). Higher homocysteine concentrations at expected ovulation were associated with a 33% increased risk of sporadic anovulation. We observed no risk associated with decreased folate concentrations, but a higher ratio of folate to homocysteine at ovulation was associated with a 10% decreased risk of anovulation.

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LIMITATIONS, REASONS FOR CAUTION: Our results are generalizable to healthy women with adequate serum folate levels. The independent influence of homocysteine should be confirmed in larger cohorts and among women with folate deficiency or increased risks of anovulation.

WIDER IMPLICATIONS OF THE FINDINGS: If these findings are confirmed, it is possible that lowering homocysteine with B-vitamins through diet or supplementation could improve ovulatory function in some women.

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Key words: folic acid / folate / homocysteine / menstrual cycle / longitudinal studies / anovulation / prospective studies

Introduction

One-carbon metabolism is essential for DNA and amino acid synthesis as well as methylation and therefore plays a crucial role in human reproduction (Laanpere et al., 2010). Integral to these biologic pathways are folate and the amino acid homocysteine, an inflammatory biomarker associated with oxidative stress and protein damage (Jakubowski, 2000; Smolders et al., 2003).

Folate and homocysteine are often studied in the context of reproductive health, particularly among women undergoing fertility treatment (Boxmeer et al., 2009; Gaskins et al., 2015). Very few studies explore the effects of folate and homocysteine on menstrual cycle characteristics and these studies focus on associations with dietary or supplemental folate, rather than serum or plasma biomarkers which may more accurately reflect individual bioavailability and metabolism (Chavarro et al., 2008; Gaskins et al., 2012; Cueto et al., 2015). In our previous work, we found higher intakes of folic acid from foods were protective against sporadic anovulation and were associated with an increase in luteal phase progesterone (Gaskins et al., 2012), but we were unable to assess biomarkers of folate status at that time. We hypothesized that higher concentrations of serum folate should similarly be protective against anovulation.

Our objective for these analyses was to examine associations between serum folate and plasma homocysteine and (i) hormone concentrations across the menstrual cycle and (ii) risk of sporadic anovulation among healthy regularly menstruating women.

Materials and Methods

Study population

The BioCycle Study (2005–2007) was a prospective cohort of 259 women recruited from upstate New York (NY), USA. These women were healthy, regularly menstruating, and aged 18–44 years. Briefly, women were excluded if they were currently using oral contraceptives or had used them within the last 3 months; were currently using other medications, vitamins or supplements (including folic acid); if they had used injectable or implantable hormonal contraceptives or intrauterine devices within the last year; if they had been pregnant in the last 6 months; or if they had any diagnoses of certain chronic conditions (e.g. menstrual and ovulation disorders, uterine fibroids, history of cancer, gastrointestinal conditions associated with malabsorption). Further details of the study design and exclusion criteria have been published (Wactawski-Wende et al., 2009).

Ethical approval

The University at Buffalo Health Sciences Institutional Review Board (IRB) approved study procedures, and served as the IRB designated by the National Institutes of Health under a reliance agreement. All participants provided written informed consent.

Data collection

Participants were followed for one ($n = 9$) to two ($n = 250$) menstrual cycles. Fasting blood samples were collected at eight clinic visits across each menstrual cycle (up to 16 samples per participant): on the second day of menstruation (1 visit), during the mid- and late follicular phase (2 visits), on the day of the luteinizing hormone (LH)/follicle stimulating hormone (FSH) peak (1 visit), on the day of predicted ovulation (1 visit), and during the early, mid, and late luteal phase (3 visits). Fertility monitors (Clearblue Easy Fertility Monitor; Inverness Medical, Waltham, MA) were used to assist in the timing of mid-cycle clinic visits (Howards et al., 2009; Mumford et al., 2011). In total, 94% of participants completed at least seven clinic visits per cycle. The majority of cycles were consecutive (only 24 cycles [9%] were not consecutive). Of the 24 non-consecutive cycles, the median time interval between cycles was 30 days (interquartile range [IQR]: 27, 45), and the mean interval was 43.5 days. The range was between 19 and 143 days. Prior to the measurement of reproductive hormones, folate, and homocysteine, serum and plasma specimens were stored in a cooler, protected from light exposure, and frozen without anticoagulant at −80°C within 90 min of the blood draws.

Participants completed baseline questionnaires and provided information on demographics and their lifestyles and health histories. Dietary information across each cycle was obtained using 24-h recalls (up to four per cycle) and analyzed using the Nutrition Data System for Research software (version 2005) developed by the Nutrition Coordinating Center of the University of Minnesota (Minneapolis, MN). Daily diaries were used to assess alcohol and tobacco use throughout each menstrual cycle (defined as any use in the 2 days prior to a clinic visit compared to none). At baseline, weight and height (used to calculate BMI, kg/m^2) were measured by trained research staff using standardized procedures.

Folate and homocysteine measurement

In 2012, serum folate (nmol/L) was measured by the University of Minnesota from blood samples that were collected on each of the eight target visits across each cycle. A competitive protein binding assay was performed with a Roche Elecsys 2010 Analyzer using the Roche Folate Gen 3 reagent (Roche Diagnostics, Indianapolis, IN); the assay detected 5-methyl tetrahydrofolate (THF), 5-formyl THF, and folic acid, which were collectively analyzed and reported as a single measure of serum folate (inter-assay coefficient of variation [CV] < 9.5%). Homocysteine (μmol/L)

was measured from EDTA plasma samples taken at three visits across the cycles (mid-follicular, expected ovulation and mid-luteal phase). Samples were analyzed at the Kaleida Center for Laboratory Medicine (Buffalo, NY) using an Immulite 2000 homocysteine competitive immunoassay (inter-assay CV < 10.4% at all concentrations). Six percent of the folate measurements and one percent of the homocysteine measurements were not available/missing. The concentrations of folate in our population ranged from 12.3 to 96.4 nmol/L (the 95th percentile was 67.2) and for homocysteine: from 2.0 to 12.9 μmol/L (across all observations from all cycles).

Reproductive hormone measurement

Total estradiol, FSH, LH and progesterone were measured from serum at the Kaleida Health Center for Laboratory Medicine using solid-phase competitive chemiluminescent enzymatic immunoassays (Specialty Laboratories Inc., Valencia, CA) on a DPC Immulite 2000 analyzer (Siemens Medical Solutions Diagnostics, Deerfield, IL). Total testosterone was measured in frozen stored samples by liquid chromatography/tandem mass spectrometry employing a Shimadzu Prominence Liquid Chromatograph (Shimadzu Scientific Instruments, Inc., Columbia, MD) with an ABSceix 5500 tandem mass spectrometer (AB SCIEX, Framingham, MA) at the University of Minnesota. Increased sensitivity was obtained by using Mobile Phase B (100% acetonitrile) with a low standard of 4 ng/dL added to the standard curve. The CV's for these tests reported by the laboratories were <10% for estradiol, <5% for LH and FSH, <14% for progesterone and <7% for total testosterone. The number of measurements missing ranged from 4 to 5% for all the hormones. For our analyses on anovulation, cycles with progesterone concentrations ≤5 ng/mL and no observed serum LH peak in the mid or late luteal phase samples (in the event that timing of progesterone measurements was too early) were considered anovulatory (Lynch *et al.*, 2014). The concentrations for each of the hormones in our population ranged from: 1.0 to 833.8 pg/mL for estradiol, 0.4 to 47.1 mIU/mL for FSH, 0.1 to 101.0 ng/mL for LH and 0.1 to 27.7 ng/mL for progesterone (across all observations from all cycles). Biomarkers were measured consecutively, within a single run, to limit analytical variability (Schisterman *et al.*, 2010).

Statistical analyses

For our descriptive tables, participant characteristics were compared across tertiles of cycle-averaged folate from the first cycle using chi-square tests for categorical variables and Kruskal–Wallis tests for continuous variables. Geometric mean concentrations of folate and homocysteine from the first cycle were compared with concentrations reported in a representative reference population, respondents to the National Health and Nutrition Examination Survey (NHANES). Folate and homocysteine concentrations met normality assumptions and thus we estimated clinic visit-specific least squares (LS) mean concentrations of the biomarkers across the cycle and compared differences in means via *t*-tests. We found that folate and homocysteine concentrations varied across the cycle. Accordingly, we used continuous, time-varying variables for folate and homocysteine in our longitudinal models. There were up to eight folate measurements per participant, per cycle. As homocysteine was only measured three times per cycle, the concentrations during each cycle phase were assigned to the other observations during the same phase for statistical analysis, in order to create a value for each of the eight visits across a cycle (e.g. the concentration measured at the mid-follicular phase was also used as an exposure on other follicular phase visits).

All hormones were log-transformed to achieve normality; effect estimates were back-transformed and interpreted as percent change in hormone concentrations associated with a one-unit increase of folate or homocysteine. Weighted linear mixed models with random intercepts for each woman were used to estimate associations between folate or homocysteine and each reproductive hormone (using data from each clinic visit across both cycles). All models for the hormone outcomes were multilevel/longitudinal and took repeated observations per woman into account (up to eight clinic visits per cycle and up to two cycles per woman). These were complete case analyses. For progesterone, we limited our models to those observations from the luteal phase in each of the cycles, as we expected low concentrations and little variation in the follicular phase; similarly, models for LH were limited to the observations around ovulation—those corresponding to the late follicular phase, LH/FSH peak and predicted ovulation.

Potential confounders were selected *a priori* from the literature and with the use of directed acyclic graphs (i.e. causal diagrams; see Greenland *et al.*, 1999). Models for folate were adjusted for age, race, BMI, cigarette use in the 2 days prior to a clinic visit, alcohol use in the 2 days prior to a clinic visit, and energy intake and fiber intake averaged for each cycle for each woman. Missing information on covariates was minimal and is presented in Table I. A quadratic term for age was added to models to ease linearity assumptions and minimize potential residual confounding. Inverse probability of exposure weights were used to control for our confounders as well as time-varying confounding by prior (from the preceding visit) folate and all prior hormone concentrations except testosterone (prior folate and homocysteine concentrations were included in the models when homocysteine was the exposure of interest) (Cole and Hernan, 2008; Moodie and Stephens, 2011).

The risk of an anovulatory cycle associated with concentrations of folate, homocysteine, or their ratio at or before ovulation was estimated using modified Poisson regression with robust errors to obtain risk ratios (RR) and 95% CI. These models accounted for multiple cycles per woman and were adjusted for the same factors previously mentioned, excepting the prior concentrations of folate, homocysteine or reproductive hormones. All statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC).

Results

Age and BMI were not distributed differently between tertiles of serum folate, but white women were more likely to be in the highest tertile of folate than in lower tertiles (77 versus 58% and 43% in the second and first tertiles, respectively; Table I). Energy and fiber intakes were highest and homocysteine concentrations were the lowest among women in the highest tertile of serum folate.

Folate concentrations in our study (geometric mean of 45.2 nmol/L for Cycle 1) were comparable to those measured among female respondents of NHANES (45.4 nmol/L) (Pfeiffer *et al.*, 2015). Geometric mean homocysteine concentrations in our population were also comparable to those reported in NHANES (BioCycle: 5.9 μmol/L; NHANES 19–30 years: 6.0 μmol/L; NHANES 31–50 years, 6.5 μmol/L) (Ganji and Kafai, 2006). We observed that folate levels were lowest during menses (compared to levels at ovulation and in the luteal phase) and homocysteine was lowest during the luteal phase when comparing mid-luteal concentrations to follicular concentrations (Supplementary Fig. SI). The median folate concentration in ovulatory cycles was 46.4 nmol/L (IQR: 37.6, 55.5) and was 44.9 nmol/L (IQR: 36.5, 55.2) in anovulatory cycles (including multiple observations per woman per cycle). For homocysteine, the concentrations were 5.8 μmol/L (IQR: 4.8, 6.9) and 6.3 μmol/L (IQR: 5.5, 7.4) for ovulatory and anovulatory cycles, respectively.

There was a significant positive association between serum folate and luteal phase progesterone; a one-unit increase in folate (nmol/L) was associated with a 1.0% increase in progesterone (95% CI: 0.4, 1.6; Table II). Higher folate concentrations were marginally associated with lower ovulatory FSH (adjusted percent change [aPC] −0.3%, 95% CI: −0.5, 0.0; $P = 0.06$; Table II).

Table I Comparison of population characteristics by tertile of average serum folate (nmol/L) from the first menstrual cycle of follow-up in the BioCycle Study (2005–2007).

Participant Characteristics	First tertile median 35.9 range 21.2–41.4 N = 87		Second tertile median 46.6 range 41.4–52.2 N = 84		Third tertile median 57.4 range 52.2–78.5 N = 88		P[a]
	n	%	n	%	n	%	
Age (years)							0.35
Median [range]	24 [18–44]		23 [18–44]		26 [18–43]		
Missing	0		0		0		
Race							<0.001
White	37	42.5	49	58.3	68	77.3	
Black	30	34.5	15	17.9	6	6.8	
Other	20	23.0	20	23.8	14	15.9	
Missing	0		0		0		
Body mass index (kg/m^2)							0.67
Underweight (<18.5)	3	3.5	2	2.4	4	4.6	
Normal (≥18.5 to <25)	51	58.6	55	65.5	52	59.1	
Overweight (≥25 to <30)	21	24.1	19	22.6	26	29.6	
Obese (≥30)	12	13.8	8	9.5	6	6.8	
Missing	0		0		0		
Cigarette use during ovulation[b]							0.07
Yes	3	3.5	8	9.8	2	2.4	
Missing	1		2		4		
Alcohol use during ovulation[b]							0.14
Yes	13	15.1	19	23.2	23	27.4	
Missing	1		2		4		
Mean energy intake (kcal)							<0.001
Median [range]	1470 [764–2793]		1524 [514–2480]		1710 [800–2850]		
	0		0		0		
Mean fiber intake (g)							<0.001
Median [range]	9.9 [4.7–31.1]		11.9 [4.7–34.2]		15.7 [7.2–36.1]		
	0		0		0		
Mean plasma homocysteine (µmol/L)							<0.001
Median [range]	6.4 [4.0–11.2]		6.2 [2.9–10.9]		5.3 [2.7–8.3]		
	0		0		0		
Prevalence of anovulatory cycles (Cycle 1)							
	7	8.1	9	10.7	8	9.1	0.832

IQR = interquartile range.
[a]Kruskal–Wallis tests are used to compare continuous variables across tertiles and chi-square tests are used for comparisons of categorical variables. A *P*-value of < 0.05 was considered statistically significant.
[b]Cigarette and alcohol use varied across the cycle in our longitudinal models of hormone concentrations; use at ovulation is presented here for brevity and was included in the anovulation models.

One-unit (µmol/L) increases in plasma homocysteine were associated with lower total estradiol and luteal phase progesterone (Table II). Plasma homocysteine was positively associated with FSH concentrations both across the cycle and at ovulation (aPC at ovulation: 2.4%, 95% CI: 0.2, 4.7; Table II).

The prevalence of anovulation was similar across serum folate tertiles for the baseline/first menstrual cycle (Table I), as well as for the second cycle (not shown). In Cycle 1, the prevalence of anovulatory cycles increased from 4.8 to 12.8% across tertiles of plasma homocysteine, but these differences were not statistically significant ($P = 0.196$, not shown). The prevalence of anovulation was not statistically different across homocysteine tertiles for the second cycle ($P = 0.662$). After adjustment for confounding, higher homocysteine concentrations at ovulation were associated with a 33% increased risk of sporadic anovulation (adjusted risk ratio [aRR]: 1.33, 95% CI: 1.12, 1.59), whereas a higher ratio of folate to homocysteine at ovulation was associated with a

Table II Percent change in hormone levels associated with unit changes in continuous (time-varying) serum folate and plasma homocysteine levels across the menstrual cycle in the BioCycle Study (2005–2007).

	Serum folate (nmol/L)[a]		Plasma homocysteine (μmol/L)[b]	
	Percent change	95% CI	Percent change	95% CI
Testosterone	0.0	−0.1, 0.1	0.5	−0.5, 1.5
Estradiol	0.0	−0.3, 0.2	**−2.3**	**−4.2, -0.3**
Ovulatory LH	−0.2	−0.6, 0.2	−1.1	−4.2, 2.1
FSH	0.1	−0.1, 0.2	**2.3**	**0.9, 3.8**
Ovulatory FSH	−0.3	−0.5, 0.0	**2.4**	**0.2, 4.7**
Luteal Progesterone	**1.0**	**0.4, 1.6**	**−6.5**	**−11.1, −1.8**

CI = confidence interval, Intervals in bold are statistically significant at $P < 0.05$.
[a]All models adjusted for age, BMI, race, cigarette use, alcohol use, energy intake, fiber intake, prior serum folate, prior hormone levels (all hormones in table except testosterone).
[b]Adjusted for factors listed in footnote above, as well as prior serum homocysteine.

Table III Risk of anovulation associated with unit changes in serum folate (nmol/L) and plasma homocysteine (μmol/L) levels in the BioCycle Study (2005–2007).

	RR[a]	95% CI
Folate level, mid-follicular phase	0.99	0.97, 1.02
Folate level, expected ovulation	1.00	0.97, 1.02
Homocysteine level, mid-follicular phase	1.17	0.98, 1.40
Homocysteine level, expected ovulation	**1.33**	**1.12, 1.59**
Ratio of serum folate to plasma homocysteine		
Ratio, mid-follicular phase	0.91	0.82, 1.02
Ratio, expected ovulation	**0.90**	**0.82, 1.00**

RR = risk ratio, CI = confidence interval. Intervals in bold are statistically significant at $P < 0.05$.
[a]All models adjusted for age, BMI, race, cigarette use, alcohol use, energy intake, fiber intake.
The median concentrations of average folate before ovulation for the first, second, and third tertile were 35.0 (range: 19.5–40.9), 46.3 (range: 40.9–51.7) and 57.7 (range: 51.7–80.3) nmol/L, respectively. For homocysteine the medians were 4.6 (range: 2.4–5.3), 6.0 (range: 5.3–6.5) and 7.4 (range: 6.6–11.5) μmol/L, respectively.

decreased risk (aRR: 0.90, 95% CI: 0.82, 1.00; Table III). Serum folate concentrations were not associated with anovulation risk.

Discussion

Our results suggest that higher plasma homocysteine concentrations are associated with both sporadic anovulation and hormonal changes that may be indicative of impaired ovulatory function. Serum folate concentrations were not consistently predictive of altered menstrual cycle characteristics, but we identified positive associations with luteal phase progesterone, an indicator of corpus luteum activity. Our identifying associations with homocysteine rather than folate, may be attributable to homocysteine concentrations being influenced by a variety of factors. However, given the healthy levels of folate in our population and our controlling for both folate and reproductive hormone levels, these findings suggest that homocysteine itself (or something that elevates homocysteine, like B12 deficiency) is affecting circulating reproductive hormone levels. To our knowledge, this is the first study to examine menstrual cycle characteristics in relation to biomarkers for folate and homocysteine.

Dietary folate or use of or supplements containing folic acid has been examined in the few studies of one-carbon metabolism and the menstrual cycle that we identified (Chavarro *et al.*, 2008; Gaskins *et al.*, 2012; Cueto *et al.*, 2015). The Danish Pregnancy Planning Study found that folic acid supplementation was associated with reduced odds of short cycle length (Cueto *et al.*, 2015). Chavarro *et al.* (2008) found that multivitamin use was protective against ovulatory infertility among healthy married women and that higher B-vitamin intakes helped explain this association. In previous work with the BioCycle Study, we assessed the impact of dietary folate and found that women with the highest intakes of synthetic folate from fortified foods had a decreased odds of sporadic anovulation and increased luteal phase progesterone compared to women with the lowest intakes (Gaskins *et al.*, 2012). In the current analyses, we did not observe associations between serum folate and sporadic anovulation which may partly be explained by the inherent measurement error in dietary recall, as we found only modest correlations between dietary folate equivalents and serum folate in our population ($\rho = 0.45$). Serum and plasma markers better address individual variation in one-carbon metabolism than dietary folate and also allowed us to investigate the role of homocysteine. Previous studies have noted folate degradation over time in stored samples (Hannisdal *et al.*, 2010) and this may have influenced our findings, though our samples were stored only for 5–7 years before folate was assayed and at colder temperatures than specimens in the study measuring degradation. Given our identifying sufficient levels of serum folate in our healthy population with adequate dietary B vitamin intake (Gaskins *et al.*, 2012), we can likely conclude that our participants were not folate deprived. This and a lack of variability in folate concentrations, may also explain our null associations between serum folate and measures of ovarian cycle function.

Three small studies evaluated changes in homocysteine levels across the menstrual cycle, but did not measure hormone concentrations or examine anovulation (De Cree *et al.*, 1999; Tallova *et al.*, 1999; Elhadd *et al.*, 2003). As in our study, these researchers found that homocysteine concentrations were lowest in the luteal phase. After controlling for potential feedback mechanisms by adjusting for hormone and folate levels, our findings of higher FSH and lower progesterone associated with higher homocysteine concentrations may indicate a dominant follicle failed to develop or that luteinization (and subsequent progesterone production) was less likely to occur in a given cycle. Support for this idea comes from studies among women undergoing fertility treatment. Twigt *et al.* (2011) also noted a positive association between FSH receptor expression and homocysteine concentrations in the granulosa cells of women undergoing fertility treatment. However, this group evaluated homocysteine in EDTA whole blood, which may lead to artificially increased homocysteine levels as storage time increases (Midttun *et al.*, 2014). Several studies show that women

undergoing fertility treatment and who had common mutations of the MTHFR gene, had reduced ovarian responsiveness or variation in the amount of recombinant FSH needed for stimulation (Thaler et al., 2006; Hecht et al., 2009; Marci et al., 2012; Liew and Gupta, 2015). Genetic variation in our population and a decreased FSH responsiveness, may also partly explain our observations.

Folate and homocysteine levels are determined by both genetic variation and diet, and homocysteine levels are higher when folate and several other B-vitamin coenzyme (B2, B6, B12) concentrations are low (Laanpere et al., 2010). Though serum measures of these vitamins were unavailable, estimated dietary intakes of these vitamins were found to be near the Recommended Dietary Allowances (Institute of Medicine, 1998). Thus, dietary B-vitamin inadequacy may not fully account for the highest homocysteine concentrations in our population. It is possible that homocysteine influences ovulatory function in response to concentrations of these B-vitamins or independent of it.

One-carbon metabolism, including folate and homocysteine, is integral to DNA and amino acid synthesis and methylation, processes which impact cellular proliferation and are of interest in the context of the menstrual cycle, as developing follicles and endometrial cells constantly grow and divide. Indeed, folate and homocysteine levels have been shown to alter cellular proliferation in animal and *in vitro* studies through pathways that involve p53 (Koury et al., 1997; Yu et al., 2013; Zhang et al., 2015). It is possible that changes in one-carbon metabolism impact the cellular division of ovarian follicle cells and consequently, ovulation and progesterone production.

There is also evidence to suggest that one-carbon metabolism is associated with inflammation (Meng et al., 2013; Abbenhardt et al., 2014). Ovulation specifically, can be viewed as an inflammatory process (Espey, 1994) and would logically be influenced by the inflammatory milieu. Homocysteine is associated with oxidative stress through the production of reactive oxygen species and nitric oxide regulation (Smolders et al., 2003). Several *in vitro* studies found that nitric oxide plays a role in luteal apoptosis/regression (Friden et al., 2000; Vega et al., 2000). Work from Wang and associates indicates that through nuclear factor kappa B transcription factor (NF-κB) pathways, homocysteine can increase monocyte chemoattractant protein-1 expression (Wang et al., 2000); this expression leads to the recruitment of macrophages—another hallmark of corpus luteum regression (Senturk et al., 1999). Homocysteine may also induce expression of NF-κB-activating inflammatory cytokines like tumor necrosis factor (TNF) (Meng et al., 2013). Control of inflammation and NF-κB activity throughout the menstrual cycle is important; researchers have noted interactions between TNF and progesterone production in human luteal cells (Chae et al., 2007) and have observed an increase in NF-κB activity and associated cytokines as progesterone levels decrease—such as the drop in levels at the end of menstruation (van der Burg and van der Saag, 1996; King et al., 2001). Our finding of lower progesterone associated with higher homocysteine may suggest involvement of mechanisms such as these. Ultimately, there are few relevant mechanistic studies with which to compare our results on homocysteine and its effect on the ovarian cycle and which would enhance our ability to make causal inferences.

This study is limited in that it can only be generalized to healthy regularly menstruating volunteers and our results should be confirmed in populations with greater variability in serum folate concentrations, as well as among women with ovulatory disorders. A strength of this study is the longitudinal measurement of serum folate, plasma homocysteine and serum hormone concentrations across multiple menstrual cycles. The use of repeated measures allowed for the control of time-varying confounding by prior hormone levels, due to fluctuations in one-carbon metabolism that are likely influenced by hormonal changes (Morris et al., 2000; Guthrie et al., 2005; Houshdaran et al., 2014). Although our supplemental figures indicate cyclic changes in one-carbon metabolism, we believe our regression findings indicate a potential role for homocysteine in influencing hormone concentrations, rather than vice versa, because we controlled for hormone and folate levels across the cycle. This is supported by studies showing that B-vitamin intake influences ovulatory functioning (Chavarro et al., 2008; Gaskins et al., 2012). Furthermore, we identified little cyclic variation in the consumption of folate among BioCycle Study participants, such that the hormone changes we associated with dietary folate levels in our previous work cannot be due to the hormone levels influencing intake (Gaskins et al., 2012; Gorczyca et al., 2016).

We were able to demonstrate that concentrations of homocysteine among healthy women with adequate folate levels may impact the ovulatory cycle, which has implications for both quality of life and fertility. Our findings suggest that homocysteine may influence the menstrual cycle directly, that homocysteine is a more sensitive marker of long term folate intake or folate status than is serum folate, or that homocysteine concentrations are a reflection of other factors such as B-vitamins levels. If other studies confirm our findings, increasing B-vitamins through diet and supplementation may offer a low-cost solution to decrease homocysteine levels and improve ovulatory function and fertility.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

K.A.M. developed the analytic plan, performed statistical analyses, and drafted and revised the article. J.W.W., J.L.M., K.C.S., A.J.G., E.H.Y., K.K., T.C.P., L.A.S., E.N.C. and S.L.M. reviewed and revised the article for important intellectual content. K.C.S., A.J.G., S.L.M. also consulted on statistical analyses. J.W.W. designed and conducted the BioCycle Study. All authors approved the final manuscript as submitted.

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Conflict of interest

None declared.

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