Homocysteine Is Lower in the Third Trimester of Pregnancy in Women with Enhanced Folate Status from Continued Folic Acid Supplementation

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Background: In many countries, current recommendations are that women take a daily 400-μg folic acid supplement from before conception until the end of the 12th week of gestation for the prevention of neural tube defects. Low folate status is associated with an increased concentration of plasma total homocysteine (tHcy), a risk factor associated with pregnancy complications such as pre eclampsia.

Methods: In a longitudinal study, we determined tHcy and corresponding folate status in 101 pregnant women at 12, 20, and 35 weeks of gestation, in 35 nonpregnant controls sampled concurrently, and in a subgroup (n = 21 pregnant women and 19 nonpregnant controls) at 3 days postpartum.

Results: Plasma tHcy was significantly lower throughout pregnancy compared with nonpregnant controls, with values lowest in the second trimester before increasing toward nonpregnant values in the third trimester. Importantly, tHcy concentrations were lower in pregnant women taking folic acid supplements than in those not, an effect that reached significance in the third trimester (5.25 vs 6.89 μmol/L; P <0.05). During the third trimester, tHcy concentrations were significantly higher in pregnant women with a history of miscarriage than in women with no previous history (7.32 vs 5.62 μmol/L; P <0.01).

Conclusions: This is the first longitudinal study to show that homocysteine concentrations increase in late pregnancy toward nonpregnant values; an increase that can be limited by enhancing folate status through continued folic acid supplementation. These results indicate a potential role for continued folic acid supplementation in reducing pregnancy complications associated with hyperhomocysteinemia.

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As a result of unambiguous evidence published over 10 years ago showing that folic acid protects against both the first occurrence and recurrence of neural tube defects (NTDs)1,2, expert committees worldwide issued folic acid recommendations. In essence, these guidelines recommend that women of child-bearing age, capable of becoming pregnant, take 400 μg/day folic acid (3–5). In general, the recommended timing of folic acid administration is from before conception until the end of the first trimester. Although the prevention of NTDs (which are malformations occurring in very early pregnancy) is clearly the focus of current policy, the importance of folate status in later pregnancy is much less clear, despite well-established evidence that maternal folate status is compromised throughout pregnancy. The issue of whether women should be supplemented with folic acid in later pregnancy is not covered by any official recommendations; therefore, practice is likely to be very variable among healthcare professionals caring for women during pregnancy.

Folate is essential for DNA and RNA biosynthesis and is required for homocysteine metabolism. Hyperhomocysteinemia is not only considered to be a strong inde-
dependent risk factor for vascular disease (6), but is also associated with pregnancy complications and adverse pregnancy outcomes (7), including NTDs (8), early pregnancy loss (9, 10), and in later pregnancy, placental abruption (11). Recently, it has been reported that, as early as 15 weeks of gestation, women who subsequently go on to have severe preeclampsia have significantly increased plasma total homocysteine (tHcy) concentrations compared with control women of similar gestational age (12). Folate is the major determinant of tHcy concentration, with well-established evidence that supplementation with folic acid has a marked homocysteine-lowering effect (13). Pregnant women have a high folate requirement because of increased folate utilization and catabolism (14); they thus might be expected to have reduced folate status and subsequent higher tHcy concentrations, particularly in the later stages of pregnancy. On the contrary, however, several studies have in fact reported that tHcy concentrations are lower during pregnancy compared with the nonpregnant state (15–17). Thus, the relationship between maternal folate status and homocysteine concentrations during pregnancy is complex. This is further compounded by the fact that no longitudinal study of homocysteine in pregnancy to date has included corresponding measures of folate status.

In the current study we assessed the role of folate status in modulating tHcy concentration during pregnancy in a longitudinal study. Specifically, we sought to address whether folic acid supplementation in later pregnancy was of potential benefit to maternal health with respect to tHcy concentrations and folate status.

**Materials and Methods**

**Participants and Study Design**

The Research Ethics Committee of the University of Ulster approved the study, and informed consent was obtained from each participant on recruitment. The study design was a longitudinal study involving healthy pregnant women and age-matched control individuals. Women attending antenatal clinics at the Jubilee Maternity Unit, Belfast City Hospital, were recruited into the study during their first antenatal visit at ~12 weeks of gestation, with age-matched nonpregnant controls randomly recruited at the same time from healthcare workers and university employees. Study participants had no previous history of thrombosis or other chronic illness. Controls were not taking any prescribed medication, including oral contraceptives, and were not taking vitamin or folic acid supplements. Patient records were checked in the postnatal period to confirm that all pregnancies remained uncomplicated. Anthropometric data on all study participants were obtained on recruitment. All study participants were asked about their use of vitamin and mineral supplements at each time-point, and any folic acid supplementation was noted.

**Sample Collection and Analysis**

Pregnant women had nonfasting blood samples collected at 12, 20, and 35 weeks of gestation. Nonpregnant controls were sampled concurrently to control for any seasonal variation. A subgroup was sampled at 3 days post partum (n = 21 pregnant and 19 nonpregnant). At each visit, blood samples were collected into tripotassium EDTA anticoagulant tubes, immediately wrapped in aluminum foil, and placed on ice until centrifugation within 3 h at 1000g for 15 min. The buffy coat was removed and aliquoted for subsequent DNA extraction. Blood for red cell folate (RCF) analysis was prepared as described by Molloy and Scott (18). Separated plasma, buffy coat, and red cell lysates were stored at −70 °C until analysis. Samples were batch-analyzed at the end of the study.

The IMx® Homocysteine assay (Abbott GmbH), a fluorescence polarization immunoassay, was used to measure tHcy concentrations (19). Plasma folate and RCF were measured by microbiological assays (17). For each participant, DNA was extracted from buffy coat samples by use of a QIAamp DNA Mini Kit (QIAGEN Ltd.). Genotyping for the 5,10-methylenetetrahydrofolate reductase; (MTHFR) C677T thermolabile mutation was performed by PCR and Hinf1 digestion as described by Frosst et al. (20).

**Statistical Analysis**

Statistical analyses were carried out with SPSS 11.0 for Windows (SPSS Inc.). Results were considered statistically significant when P was <0.05. Analyses of the data on tHcy, plasma folate, and RCF revealed a skewed distribution; consequently, values were transformed logarithmically before statistical analyses to approximate gaussian distribution. Genotype frequency differences between the study groups were assessed by the χ² test. Data were analyzed by repeated-measures ANOVA using the general linear model, with pregnant/nonpregnant as the between-subject factor and MTHFR genotype, body mass index [BMI; weight (kg)/height (m)²], and smoking as covariates. Where there was a significant effect of time, specific comparisons between time-points were made with the Bonferroni correction. Repeated-measures ANOVA assuming constant correlation (sphericity) was used to assess the effects of time and group. Fixed effects were included for group, time, the interaction, and the covariates. The use of alternative correlation structures did not affect the estimates of confidence intervals (CIs). Multiple regression analysis was used to examine the relationship between history of miscarriage and plasma homocysteine, allowing for possible confounding factors.

**Results**

**Study Compliance and Participant Characteristics**

Of 120 pregnant women initially recruited, 101 successfully completed the study to the 35-weeks of gestation time-point. None of the women developed preeclampsia...
or symptoms of venous thromboembolic events, and all women delivered healthy babies. Of the 41 controls originally recruited, 35 full data sets were suitable for inclusion in the final analysis. In parallel with 21 mothers sampled at 3 days post partum, 19 randomly selected nonpregnant controls were sampled. There was no significant difference in age, BMI, frequency of smokers and nonsmokers, or MTHFR C677T polymorphism frequencies between pregnant women and nonpregnant controls at time of recruitment, as shown in Table 1.

PLASMA tHcy CONCENTRATION AND FOLATE STATUS

The plasma tHcy, plasma folate, and RCF concentrations are shown in Table 1 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue3/.

Repeated-measures ANOVA applied to the tHcy data showed an overall difference between pregnant women and nonpregnant controls ($F = 56.271; P < 0.001$). Homocysteine concentrations were significantly lower in pregnant women than in nonpregnant controls at 12 ($P < 0.001$; ratio = 0.60; CI, 0.54–0.67), 20 ($P < 0.001$; ratio = 0.61; CI, 0.57–0.72), and 35 weeks of gestation ($P = 0.001$; ratio = 0.80; CI, 0.70–0.91), whereas by 3 days post partum there was no difference between the two groups. Repeated-measures ANOVA applied to the RCF and plasma folate data showed an overall difference between pregnant women and nonpregnant controls: $F = 15.789 (P < 0.001)$ and $F = 5.077 (P < 0.05)$ for RCF and plasma folate, respectively. RCF was significantly higher in pregnant women than in nonpregnant controls at 12 ($P < 0.001$; ratio = 1.30; CI, 1.10–1.53), 20 ($P < 0.001$; ratio = 1.43; CI, 1.24–1.65), and 35 ($P < 0.05$; ratio = 1.24; CI, 1.04–1.46) weeks of gestation but not at 3 days post partum. Plasma folate was higher in pregnant women than in nonpregnant controls at 12 ($P < 0.001$; ratio = 1.96; CI, 1.38–2.44) weeks only, an effect largely driven by folic acid supplement use during early pregnancy.

Repeated-measures ANOVA applied to the tHcy and folate data showed that there was no significant change in tHcy concentration or folate status over time in the nonpregnant control group. However, in the pregnant group, there was a significant effect of gestational time on tHcy concentration, plasma folate, and RCF. Plasma tHcy concentrations were significantly lower at week 12 compared with week 35 ($P < 0.001$; ratio = 0.78; CI, 0.72–0.85) and at week 20 compared with week 35 ($P < 0.001$; ratio = 0.78; CI, 0.72–0.82). RCF concentrations were significantly higher at week 12 compared with week 35 ($P < 0.05$; ratio = 1.13; CI, 1.02–1.26) and at week 20 compared with week 35 ($P < 0.05$; ratio = 1.09; CI, 1.02–1.21), whereas plasma folate concentrations were significantly higher at week 12 compared with week 20 ($P < 0.001$; ratio = 2.03; CI, 1.77–2.33) and week 35 ($P < 0.001$; ratio = 2.15; CI, 1.77–2.60).

Multiple regression analysis indicated a significant effect of history of miscarriage ($n = 25$; confirmed on the basis of examination of the medical records) compared with no history of miscarriage ($n = 76$) on plasma tHcy at 35 weeks (Fig. 1), a relationship that was not confounded by use of folate supplements, BMI, smoking, or MTHFR genotype ($P < 0.01$; ratio = 0.80; CI, 0.68–1.05).

EFFECT OF FOLIC ACID SUPPLEMENTATION IN PREGNANCY

Repeated-measures ANOVA showed that, as expected, pregnant women who took a folic acid supplement had significantly higher RCF and plasma folate at 12, 20, and 35 weeks of gestation compared with those who did not (Fig. 2). Repeated-measures ANOVA also showed that plasma tHcy concentrations were lower in pregnant women taking a folic acid supplement than in those

| Table 1. Characteristics of pregnant women and nonpregnant controls at recruitment. |
|---------------------------------|------------------|------------------|----------|------------------|----------|
| Age, years | Pregnant (n = 101) | Nonpregnant (n = 35) | $\chi^2$ | $t$ | $P$ |
| Mean (SD) | 28.8 (5.6) | 27.2 (5.7) | 1.508$^a$ | NS$^b$ |
| Range | 17–41 | 20–42 | | |
| Mean (SD) BMI, kg/m$^2$ | 25.3 (4.7) | 24.4 (4.1) | 1.074$^a$ | 25.3 (4.7) |
| Smokers, % | 30 | 42.9 | 2.031$^c$ | NS |
| MTHFR genotype, % | | | | |
| CC | 45.5 | 54.3 | | |
| CT | 41.6 | 28.6 | 1.906$^c$ | NS |
| TT | 12.9 | 17.1 | | |
| Reporting folic acid supplement use, % | | | | |
| First trimester | 90 | | | |
| Second trimester | 27 | | | |
| Third trimester | 30 | | | |

$^a$ Independent t-test.

$^b$ NS, not significant.

$^c$ $\chi^2$ test.
women not taking a supplement, an effect that was statistically significant at 35 weeks ($P < 0.001$; ratio $0.78$; CI, 0.72–0.86), as shown in Fig. 2. The homocysteine-lowering effect of folic acid supplementation in the third trimester was observed irrespective of whether women had a reported history of miscarriage (results not shown).

**Discussion**

In the current longitudinal study, we observed, as others have shown (15–17), lower tHcy concentrations in the pregnant state. Plasma tHcy concentrations were lower during each trimester of pregnancy than in nonpregnant controls and by 3 days post partum were not significantly different between the two groups. Several explanations have been proposed for the lower tHcy concentrations in pregnancy shown here and elsewhere, including hemodilution, decreased albumin concentrations during pregnancy, or a relationship with maternal folic acid supplementation during pregnancy (15, 16). Recently, however, Murphy et al. (17), in a study that was also of longitudinal design, provided good evidence that the pregnancy-related decrease in tHcy concentration was not explained by any of these factors, or indeed by changes in renal hemodynamics (21), as suggested by others (22). Moreover, Powers et al. (23) recently reported that although renal handling of homocysteine during normal pregnancy may contribute to the changes observed in plasma homocysteine, it was unlikely to completely explain these changes. Clearly, the mechanism behind the lowering of tHcy concentration in pregnancy is currently the subject of much debate, as is the question of when tHcy concentrations return to nonpregnant values. A recent report by Murphy et al. (24) indicated that by onset of labor tHcy concentrations were similar to preconception ranges in women not taking folic acid supplements. In the current study, the critical timing of sampling enabled the pattern of change in tHcy concentration throughout pregnancy to
be observed, a pattern that may have been missed by previous studies, which sampled earlier in the third trimester (17). The pattern indicated by our results shows that tHcy concentrations are lowest during the second trimester of pregnancy and increase in the second half of the third trimester to return toward nonpregnant concentrations. To the best of our knowledge, no hormone or marker of renal function conforms to the pattern of change in tHcy concentration shown here. Estradiol concentration, for example, increases linearly throughout pregnancy (25), and recent evidence suggests that its association with homocysteine is evident only up to 20 weeks of gestation (26). It is likely, therefore, that the lower tHcy concentration in the pregnant state is related to altered maternal amino acid metabolism driven by fetal requirements; however, the precise mechanism remains unclear.

Our results show that pregnant women taking folic acid supplements had lower tHcy concentrations than those women not taking a supplement, an effect that reached significance in the third trimester. For ethical reasons, a placebo-controlled intervention study was not feasible and, therefore, our approach was observational, with supplement usage established simply by asking participants to declare at each visit whether they were using folic acid supplements. We can confirm the validity of the classification of participants into supplement users and nonusers on the basis of their reported usage because there was a marked difference in folate status between those women reporting supplement use compared with nonusers. We show that folate status is a more powerful determinant of homocysteine in the third trimester than in the first and second trimesters, possibly suggesting that mechanisms regulating tHcy metabolism in the early stages of pregnancy may mask endogenous hyperhomocysteinemia, whereas in the later stages of pregnancy tHcy concentrations may be more reflective of nonpregnant values. Our data suggest that the physiologic increase in tHcy concentration in the latter half of the third trimester shown here can be modulated by folic acid supplementation.

In the current study, pregnant women with a previous history of one or more miscarriages were found to have significantly higher tHcy concentrations at 35 weeks of gestation compared with those women with no history of miscarriage. This relationship remained even after controlling for possible confounding factors, such as use of folic acid supplements; MTHFR C677T polymorphism, the most common genetic cause of increased tHcy; and smoking. The authors of recent metaanalysis have reported that although homozygosity for the MTHFR polymorphism represents a small increase in the risk of early recurrent pregnancy loss, it is a less apparent risk factor than increased homocysteine (10). When the effect of this polymorphism was examined in the current study, we found an increased prevalence of the TT genotype among those women with a history of miscarriage compared with those without (24% vs 10%), although this was not statistically significant.

The current study is the first longitudinal study to examine both tHcy concentration and folate status in normal pregnancy. The results obtained are consistent with previous studies showing that tHcy concentrations are lower during pregnancy. The current study shows that tHcy concentrations are lowest in the second trimester before increasing toward nonpregnant values in the third trimester. Although the precise mechanism of decreased tHcy concentration during pregnancy is not known, it is most likely to be regulated primarily by fetal amino acid requirements and utilization. Importantly, our results suggest that continued folic acid leads to enhanced folate status and correspondingly in a lowering of tHcy concentrations, an effect that is most marked in the third trimester. Therefore, continuing folic acid supplementation for the duration of the pregnancy, beyond 12 weeks of gestation as currently recommended, may help to prevent late pregnancy complications attributed to hyperhomocysteinemia, such as preeclampsia. These findings have particular relevance for many countries (including most European countries) in which there is no mandatory folic acid fortification policy and highlight the need for a randomized controlled trial to determine the effects of continued folic acid supplement usage in the second and third trimesters on pregnancy outcome.

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References


