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Genetic and Lifestyle Variables Associated with Homocysteine Concentrations and the Distribution of Folate Derivatives in Healthy Premenopausal Women

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Abstract

Background—Low folate and high homocysteine (Hcy) concentrations are associated with pregnancy-related pathologies such as spina bifida. Polymorphisms in folate/Hcy metabolic enzymes may contribute to this potentially pathogenic biochemical phenotype.

Methods—The study comprised 26 Caucasian and 23 African-American premenopausal women. Subjects gave fasting blood samples for biochemical phenotyping and genotyping. Total Hcy (tHcy) and both plasma and red blood cell (RBC) folate derivatives [i.e. tetrahydrofolate (THF), 5-methylTHF (5-MTHF), and 5,10-methenylTHF (5,10-MTHF)] were measured using stable isotope dilution liquid chromatography, multiple reaction monitoring, mass spectrometry. Eleven polymorphisms from nine folate/Hcy pathway genes were genotyped. Tests of association between genetic, lifestyle, and biochemical variables were applied.

Results—In African American women, tHcy concentrations were associated ($p < 0.05$) with total RBC folate, RBC 5-MTHF, B₁₂, and polymorphisms in *methionine synthase (MTR)* and *thymidylate synthase (TYMS)*. In Caucasian women, tHcy concentrations were not associated with total folate levels, but were associated ($p < 0.05$) with RBC THF, ratios of RBC 5-MTHF: THF, and polymorphisms in *5,10-methylenetetrahydrofolate reductase (MTHFR)* and *MTR*. In African Americans, folate derivative levels were associated with smoking, B₁₂, and polymorphisms in *MTR*, *TYMS*, *methionine synthase reductase (MTRR)*, and *reduced folate carrier1 (RFC1)*. In

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Caucasians, folate derivative levels were associated with vitamin use, B₁₂, and polymorphisms in *MTHFR*, *TYMS*, and *RFC1*.

Conclusions—Polymorphisms in the folate/Hcy pathway are associated with tHcy and folate derivative levels. In African American and Caucasian women, different factors are associated with folate/Hcy phenotypes and may contribute to race-specific differences in the risks of a range of pregnancy-related pathologies.

Keywords

Genetics; folate; homocysteine; women; reproductive age; spina bifida risk

Introduction

Low folate and high homocysteine (Hcy) concentrations are associated with pregnancy complications such as low birth weight infants, preterm birth, preeclampsia, stillbirth, and recurrent pregnancy loss (Vollset et al., 2000; D'Uva et al., 2007). They have also been associated with congenital abnormalities such as neural tube defects (NTDs), clubfoot, heart defects, limb deficiencies, cleft lip/palate, and Down syndrome (Czeizel, 1998; James et al., 1999; Vollset et al., 2000; Little et al., 2008). Moreover, inadequate maternal folate status is an established risk factor for spina bifida (Mitchell et al., 2004), and maternal periconceptional folic acid supplementation reduces the risk of spina bifida and other adverse pregnancy outcomes listed above (Czeizel and Dudas, 1992; Yang et al., 1997; Bukowski et al., 2009; Timmermans et al., 2009). Further, in the United States mandatory folic acid food fortification was introduced in 1998 in order to improve the folate status of reproductive age women. Subsequently, significantly increased folate and decreased Hcy concentrations and reductions in the prevalence of folate deficiency (<7 nmol/L, in plasma) and hyperhomocysteinemia (>13 μmol/L) were observed in the general population of the US (Jacques et al., 1999). In addition, mandatory folic acid fortification in the US was also followed by a decline in the prevalence of spina bifida by approximately 20% (Honein et al., 2001; Williams et al., 2002).

Suboptimal folate metabolism affects methylation and nucleotide synthesis, functions that are supported by the folate/Hcy pathway (Stover, 2004). Concentrations of folate and Hcy can be affected by diet, lifestyle, and genetic polymorphisms of the enzymes that control folate/Hcy metabolism. Many studies have been undertaken to characterize the biochemical consequences of such polymorphisms. Most of the previous studies measured only total folate in red blood cells (RBCs), plasma, or serum. However, absolute levels of the key RBC folate derivatives: tetrahydrofolate (THF), 5-methylTHF (5-MTHF), and 5,10-methenylTHF (5,10-MTHF) (Figure 1) may be more sensitive indicators of the role of genetic factors. As particular combinations of genetic variants and/or folate/Hcy phenotypes may be indicative, at least in part, of an individual woman's risk of pregnancy complications, we assessed the relationships between eleven polymorphisms in nine genes and folate/Hcy phenotypes, in a population of premenopausal women, using a stable isotope dilution liquid chromatography, multiple reaction monitoring, mass spectrometry (LC/MRM/MS) method to measure plasma and RBC 5-MTHF, THF, and 5,10-MTHF (Huang et al., 2008) as well as total Hcy (tHcy) (Huang et al., 2007). These analyses were undertaken in premenopausal African American

and Caucasian women because the prevalence of several folate related pregnancy complications differ in these two groups.

The polymorphisms, selected on the basis of previously published associations with folate or Hcy levels and/or risk of spina bifida, were: *5,10-methylenetetrahydrofolate reductase* (*MTHFR*) 677C>T (rs1801133); *MTHFR* 1298A>C (rs1801131); *cystathionine β synthase* (*CBS*) 844ins68; *methionine synthase* (*MTR*) 2756A>G (rs1805087); *methionine synthase reductase* (*MTRR*) 66A>G (rs1801394); *thymidylate synthase* (*TYMS*) 1494del6 (rs16430); *TYMS* 5' variable number of tandem repeat (VNTR); *dihydrofolate reductase* (*DHFR*) c. 86+60_78; *reduced folate carrier1* (*RFC1*) 80A>G (rs1051266), also known as *solute carrier family 19 member 1* (*SLC19A1*); *methylenetetrahydrofolate dehydrogenase 1*, *methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase* (*MTHFD1*) 1958G>A (2236225); and *serine hydroxymethyltransferase 1* (*SHMT1*) 1420C>T (rs1979277) (Jacques et al., 1996; Harmon et al., 1996; Harmon et al., 1999; Wilson et al., 1999; Tsai et al., 2000; Chen et al., 2001; Gaughan et al., 2001; Brody et al., 2002; Gaughan et al., 2002; Trinh et al., 2002; Doolin et al., 2002; Morin et al., 2003; De Marco et al., 2003; Volcik et al., 2003; Johnson et al., 2004; Kealey et al., 2005; Ulvik et al., 2007; Stanislawski-Sachadyn et al., 2008a; Stanislawski-Sachadyn et al., 2009).

Materials and Methods

Study Subjects

Premenopausal female subjects were recruited by advertisement from staff and students at the University of Pennsylvania School of Medicine from January 9, 2007 to July 26, 2007. This study was designed to recruit a similar number of subjects who self-reported as Caucasians and African Americans. Potential study subjects were excluded if they had a major medical condition (e.g., autoimmune disease), used an anti-folate medication or disease modifying anti-rheumatoid drug, or were pregnant. The study was approved by the Institutional Review Board of the University of Pennsylvania School of Medicine, and all subjects provided informed consent. The analyses presented here are based on values obtained at the first of two visits, during which subjects provided a fasting blood sample in the morning and completed a short, in-person interview that included questions related to use of alcohol and smoking status.

Laboratory Methods

THcy and both plasma and RBC folate derivatives were measured using LC/MRM/MS as previously described (Huang et al., 2007, 2008). The measured folate derivatives were 5-MTHF, THF, and 5,10-MTHF. The last of these represents the sum of 5,10-MTHF together with 5-formylTHF and 10-formylTHF, both of which are converted to 5,10-MTHF through acid conversion.

Vitamin B₁₂ levels were measured by using Immulite 2000 Vitamin B₁₂ Assays (Diagnostic Products Corporation, Los Angeles, CA).

Genotyping

DNA was extracted from whole blood using the QIAamp DNA Mini Kit (Qiagen, Santa Clarita, CA). TaqMan genotyping methods were previously described for *MTHFR* 677C>T and 1298A>C (Lu et al., 2008), and for *MTR* 2756A>G and *MTRR* 66A>G (Summers et al., 2008). Size difference PCR assays for *CBS* 844ins68 and *DHFR* c.86+60_78 have also been previously described (Summers et al., 2008). The published *TYMS* 1494del6 assay (Summers et al., 2008) was used except for the use of modified primer sequences: forward (5'-AATTTTACAAGCTATTCCCTCA-3') and reverse (5'-TGGACGAATGCAGAACACTT-3'), and separation using 4% Super Fine Resolution agarose (Amresco, Solon, Ohio) gels run with 1x TBE. The size difference PCR assay for *TYMS* 5' VNTR was previously described (Brown et al., 2004a) except that 2.5U AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA) and 10% DMSO were used.

TaqMan assays were developed for *MTHFD1* 1958G>A, *RFC1* 80A>G, and *SHMT1* 1420C>T genotyping. Forward and reverse primers for the *MTHFD1* assay were (5'-GTTTGCCAACATCGCACAT-3') and (5'-CATACAGTCTATTCACTGGTTTTGC-3') with 75uM FAM labeled probe (5'-TGCAGACCGGATC-3') and 37.5uM Vic labeled probe (5'-AGACCAGATCGCACT-3') (Applied Biosystems). The sequences of forward and reverse primers as well as FAM and VIC probes for the *RFC1* assay were previously published by Skibola et al. (Skibola et al., 2004) and the assay used 50uM FAM labeled probe and 100uM Vic labeled probe. Forward and reverse primers for the *SHMT1* assay were (5'-AGGAGAGACTGGCAGGGGAT-3') and (5'-CATCCATCTCTCAGGTGGGG-3') with 75uM FAM labeled probe (5'-CGCCTCTCTTTC-3') and 55uM Vic labeled probe (5'-TCGCCTCTTCTTC-3') with the Fam probe sequence previously published by Skibola et al. (Skibola et al., 2002). PCRs for the above three assays were performed with an initial incubation at 95°C for 10 min, followed by 60 cycles of assay specific denaturation and extension/5' nuclease steps. These were: 95°C for 15s and 55°C for 50s for *MTHFD1*; 95°C for 30s and 63°C for 60s for *RFC1*; and 95°C for 20s and 57°C for 45s for *SHMT1*.

Statistical Methods

Descriptive analyses of variables in the study included counts and proportions for discrete variables, and means and standard deviations for continuous variables. Body mass index (BMI) was calculated as: $\text{weight(kg)}/[\text{height(m)}]^2$, and total RBC folate as the sum of RBC 5-MTHF, THF, and 5,10-MTHF. Given their relationship as substrate and product, the ratio of RBC 5-MTHF: THF provides precise information about the relative proportions of these two analytes. This enhances analyses of the likely relationship of folate distribution to variables of interest. For THF, samples with non-detectable levels (n=4) were assigned a value corresponding to the lower limit of quantitation (4.5 nmol/L) for the THF assay. This allowed both absolute THF concentrations and the ratios of RBC 5-MTHF: THF to be analyzed as continuous variables. In contrast, RBC 5,10-MTHF was not detected in over half of the samples and was treated as a dichotomous (detectable/not detectable) variable.

Hardy-Weinberg equilibrium was evaluated for each polymorphism within each group. Differences in genotype distributions between the two groups were tested by Fisher's exact

test. Simple linear regression analyses were performed with tHcy and various forms of folate as the outcome measure. The coefficient of determination (R^2) estimated from these models was used to assess the proportion of variation in the dependent variable that was explained by each predictor variable. The significance of the association between each predictor variable was assessed using the t-statistic or Fisher's exact test. All analyses were performed separately by groups using SAS version 9.1 (SAS Institute, Inc, Cary, NC). Unadjusted P values <0.10, indicative of trends, are presented in tabular form. P values <0.05 were considered to be statistically significant and of potential interest and were evaluated in the context of biological plausibility. Given the relatively small sample size and the number of comparisons that were performed, these analyses should be considered exploratory and interpreted in that context.

Results

Subject Characteristics

Out of 49 study subjects, 26 (53%) self-reported as Caucasian and 23 (47%) self-reported as African American. The characteristics of the study subjects at the time of the first study visit and the observed genotype distributions are summarized for each of the above groups in Tables 1 and 2. Since several predictor variables were distributed differently in African Americans and Caucasians, all analyses were performed separately for each group. As the *MTHFR* 677TT homozygous genotype is known to have a profound effect on folate/Hcy phenotype, and only five such homozygotes were observed (all in Caucasians, Table 2), individuals with this genotype were dropped from all analyses except those explicitly examining the effects of the *MTHFR* 677T variant.

Genotype Distributions

The genotype distributions for *MTHFR* 677C>T, *MTRR* 66A>G, *CBS* 844ins68, *TYMS* 1494del6, and *MTHFDI* 1958G>A were significantly different between African American and Caucasian women (Table 2). However, with the exception of *MTHFDI* 1958G>A in Caucasians, all genotypes were in Hardy-Weinberg equilibrium within each of the groups.

Associations with Biochemical Phenotypes

The following sections summarize the associations of the various biochemical phenotypes with each other and with lifestyle and genetic factors. Given the large number of comparisons made, the results section focuses on associations with unadjusted P values <0.05. However, for completeness the results of all analyses with P values <0.10 are presented in Tables 3–7.

Lifestyle Factors

Smoking—African American smokers had mean total RBC folate concentrations that were 380.1 nmol/L lower than nonsmokers (625.1 vs. 1005.2 nmol/L, $p=0.038$, Table 3). This difference was largely attributable to differences in the mean RBC 5-MTHF levels of smokers and non-smokers (609.2 vs. 984.6 nmol/L, respectively, $p=0.038$). Similar relationships were observed in Caucasians but were not statistically significant.

Vitamin Use—In Caucasian women, vitamin users had higher mean folate values than non-users: total RBC folate levels (1398.6 vs. 1072.1 nmol/L, $p=0.013$), RBC 5-MTHF (1355.8 vs. 1028.9 nmol/L, $p=0.011$), and plasma 5-MTHF (67.2 vs. 43.5 nmol/L, $p=0.022$). Similar associations were not observed in African American women.

Biochemical Variables

Relationships between Hcy and folate derivatives—In African Americans, tHcy concentrations were significantly inversely associated with total RBC folate ($p=0.022$, Table 4) and RBC 5-MTHF levels ($p=0.022$) and positively associated with plasma 5-MTHF levels ($p=0.028$). In Caucasians, the ratio of RBC 5-MTHF: THF was inversely related to tHcy concentrations ($p=0.009$). RBC THF levels in Caucasians were positively associated with tHcy concentrations ($p=0.010$), which is in contrast to the inverse trend in African Americans.

Relationships among the folate derivatives—African Americans with detectable levels of RBC 5,10-MTHF had THF levels that were 13.8 nmol/L higher than in those with undetectable levels of 5,10-MTHF (31.7 vs. 17.9 nmol/L, $p=0.037$). This observation suggests that measurable quantities of 5,10-MTHF are accompanied by relatively high THF concentrations and that these two analytes have at least some degree of shared metabolic control. In African Americans, RBC 5-MTHF levels were positively associated with RBC THF levels ($p=0.017$). In addition, plasma 5-MTHF levels were positively associated with total RBC folate concentrations ($p<0.0001$), in particular with RBC 5-MTHF levels ($p<0.0001$), and the ratio of RBC 5-MTHF: THF ($p=0.001$). In Caucasians, plasma 5-MTHF levels were positively associated with total RBC folate concentrations ($p=0.017$), and in particular with RBC 5-MTHF levels ($p=0.015$), a finding that was similar to that observed in African Americans.

B₁₂ Levels—B₁₂ is the cofactor for MTR, the enzyme that remethylates Hcy. In African Americans, B₁₂ levels were inversely associated with tHcy concentrations ($p=0.049$) and positively associated with RBC THF levels ($p=0.028$). In Caucasians, there was a positive association between B₁₂ and RBC THF levels ($p=0.018$), similar to the relationship seen in African Americans.

Genetic Associations with tHcy Concentrations

African American *TYMS* 1494del6 del/del homozygotes had 2.3 $\mu\text{mol/L}$ higher tHcy levels compared to insertion carriers (10.2 vs. 7.9 $\mu\text{mol/L}$, $p=0.023$, Table 5). African American *MTR* 2756AA homozygotes had 2.7 $\mu\text{mol/L}$ higher tHcy levels than 2756G carriers (10.4 vs. 7.7 $\mu\text{mol/L}$, $p=0.006$). There were no Caucasian *MTR* 2756GG homozygotes. However, Caucasian *MTR* 2756AA homozygotes had 2.0 $\mu\text{mol/L}$ higher tHcy concentrations than 2756AG heterozygotes (9.6 vs. 7.6 $\mu\text{mol/L}$, $p=0.017$, Table 6). In Caucasians, *MTHFR* 677TT homozygotes had 3.2 $\mu\text{mol/L}$ higher tHcy concentrations than 677C carriers (12.2 vs. 9.0 $\mu\text{mol/L}$, $p=0.012$). After exclusion of *MTHFR* 677TT homozygotes Caucasian *MTHFR* 1298C carriers had mean tHcy concentrations that were 1.8 $\mu\text{mol/L}$ higher than 1298AA homozygotes (9.7 vs. 7.9 $\mu\text{mol/L}$, $p=0.031$).

Genetic Associations with Folate Derivatives

MTR—African American *MTR* 2756AA homozygotes had total RBC folate concentrations that were 286.4 nmol/L lower than 2756G carriers (777.2 vs. 1063.6 nmol/L, $p=0.042$, Table 5); this difference was attributable to mean RBC 5-MTHF concentrations that were 280.6 nmol/L lower (760.7 vs. 1041.3 nmol/L, $p=0.043$). *MTR* 2756AA homozygotes also had 14.1 nmol/L lower plasma 5-MTHF than 2756G carriers (25.5 vs. 39.6 nmol/L, $p=0.049$). Although Caucasian *MTR* 2756AA homozygotes had increased tHcy, similar to African Americans with this genotype, they did not have significantly different levels of folate derivatives.

MTRR—Although African American *MTRR* 66G carriers had levels of RBC 5-MTHF that were higher and THF levels that were lower, neither of these differences were statistically significant. However, the differences in the above analytes were sufficient for 66G carriers to have significantly higher ratios of RBC 5-MTHF: THF compared to 66AA homozygotes (59.3 vs. 45.4, $p=0.028$), indicating that the polymorphism is associated with the *proportional distribution* of these two key forms of folate.

MTHFR 677C>T—In Caucasians, total RBC folate concentrations were similar for all *MTHFR* 677 genotypes, *MTHFR* 677TT homozygotes had mean RBC 5-MTHF concentrations that were 426.3 nmol/L lower (696.0 vs. 1122.3 nmol/L, $p=0.007$, Table 6) and mean RBC THF levels that were 419.7 nmol/L higher (457.2 vs. 37.5 nmol/L, $p<0.0001$) compared to *MTHFR* 677C carriers. Furthermore, the mean ratio of RBC 5-MTHF: THF was markedly lower in *MTHFR* 677TT homozygotes (5.4 vs. 42.9, $p=0.0004$). RBC 5,10-MTHF was detectable in 4 out of 5 *MTHFR* 677TT homozygotes and the mean of the detectable concentration was 149.4 nmol/L, well above the means of detectable levels in the 677CC and CT genotypes (9.8 and 9.7 nmol/L, respectively, Table 7).

MTHFR 1298A>C—Caucasian *MTHFR* 1298C carriers had 27.0 nmol/L higher RBC THF levels than 1298AA homozygotes (49.1 vs. 22.1 nmol/L, $p=0.046$), although RBC 5-MTHF levels were not statistically different. However, the above folate analytes were sufficiently different such that 1298C carriers had lower ratios of RBC 5-MTHF: THF than 1298AA homozygotes (33.4 vs. 55.6, $p=0.008$).

TYMS—African American *TYMS* 5' VNTR 2R carriers had ratios of RBC 5-MTHF: THF that were higher than 3R/3R homozygotes (58.5 vs. 41.3, $p=0.010$). In Caucasians, *TYMS* 1494del6 deletion carriers had plasma 5-MTHF levels that were 21.7 nmol/L higher than ins/ins homozygotes (57.5 vs. 35.8 nmol/L, $p=0.030$).

RFC1—African American *RFC1* 80G carriers had 10.2 nmol/L lower RBC THF levels than 80AA homozygotes (15.5 vs. 25.7 nmol/L, $p=0.007$); however RBC 5-MTHF levels were not significantly different. Nevertheless, 80G carriers had higher ratios of RBC 5-MTHF: THF compared to 80AA homozygotes (58.0 vs. 39.3, $p=0.003$). There was a similar observation in Caucasians, in whom *RFC1* 80G carriers had significantly higher ratios of RBC 5-MTHF: THF than 80AA homozygotes (49.5 vs. 26.5 nmol/L, $p=0.012$).

Discussion

Low folate and high tHcy concentrations have been associated with many pathologies, ranging from pregnancy related complications and congenital abnormalities to cardiovascular diseases (Lucock, 2000). Pregnant Caucasian women with suboptimal folate status have an eight fold increased risk of having a baby affected with spina bifida (Molloy et al., 1998). However, the finding two decades ago that periconceptional supplementation with folic acid can prevent up to 70% of spina bifida cases (MRC Vitamin Study Research Group, 1991) suggested that intervention with B vitamins can correct the aspect of folate/Hcy phenotype that contributes to spina bifida etiology. Subsequent studies have confirmed the utility of folic acid in reducing the incidence of spina bifida (Berry et al., 1999). Nevertheless an individual woman's risk for poor pregnancy outcome is multifactorial and complex and the component that may be attributable to sub-optimal folate/Hcy metabolism is likely impacted by both genetic and lifestyle factors. Clues to the nature of such factors may be derived from populations with different levels of risk for spina bifida and other conditions. African Americans have much lower rates of spina bifida than Caucasians (Feuchtbaum et al., 1999) though the incidence of other pathologies, such as preeclampsia (Eskenazi et al., 1991), low birth weight infants, and preterm birth (Heron et al., 2010), are higher. A detailed investigation comparing the effect of folate-related genotypes and lifestyle factors on biochemical phenotypes in African American and Caucasian women of reproductive age could provide insight into the different levels of pathology-specific risk detailed above.

The laboratory methods used in this study have allowed multiple analytes (tHcy, plasma 5-MTHF, RBC 5-MTHF, RBC THF, and RBC 5,10-MTHF) to be evaluated in the context of both genetic and lifestyle variables in premenopausal African American and Caucasian women. It has been possible to delineate the impact of those variables on folate/Hcy phenotype with an emphasis on specific forms of folate that occupy key positions within the metabolic pathway (i.e. forms that support important functions such as reducing tHcy concentrations, methylation, and nucleotide synthesis). The precision of these methods minimizes measurement error and has allowed us to discern relationships between variables using relatively small numbers of study subjects.

The *MTHFR* 677C>T polymorphism is the major genetic determinant of tHcy and folate levels with the 677TT genotype being associated with relatively high tHcy concentrations, especially when folate levels are low (Jacques et al., 1996; Harmon et al., 1996). Furthermore, the *MTHFR* 677TT genotype is associated with increased risk for NTDs (Whitehead et al., 1995; van der Put et al., 1995) and a meta-analysis has shown that this genotype confers risk at the level of the embryo as well as the mother (Botto and Yang, 2000). In addition, there is evidence that the 677CT heterozygous genotype confers risk (Kirke et al., 2004). In our study Caucasian *MTHFR* 677TT homozygotes had higher tHcy concentrations than 677C carriers and had RBC folate distributions characterized by relatively high THF and 5,10-MTHF and relatively low 5-MTHF levels. These quantitative changes in analyte concentrations mandated ratios of RBC 5-MTHF: THF that were much lower in *MTHFR* 677TT homozygotes than in 677C carriers. The above biochemical observations are consistent with a study by Bagley and Selhub (Bagley and Selhub, 1998) in

which RBCs from 677TT homozygotes were shown to have relatively low 5-MTHF and high formylated THF levels.

In addition to the *MTHFR* 677TT genotype, other folate-related genotypes (i.e. *MTR* 2756G carriers, both *MTRR* 66A carriers and 66GG, *MTHFD1* 1958AA, *RFC1* 80GG, and *DHFR* c.86+60_78 del/del) (Wilson et al., 1999; Doolin et al., 2002; Brody et al., 2002; De Marco et al., 2003; Johnson et al., 2004) have been shown to confer risk for NTDs via the maternal genotype. In our study, genotype distributions for five of the eleven polymorphisms under test were significantly different between African American and Caucasian women. Of the above risk-associated genotypes Caucasians had higher frequencies for *MTHFR* 677TT and *MTHFD1* 1958AA while the other genotype frequencies were not significantly different between African Americans and Caucasians. In Caucasians, the *MTHFD1* 1958G>A polymorphism was not in Hardy-Weinberg equilibrium. This has been observed in other control populations, such as in the Irish (Parle-McDermott et al., 2006).

Polymorphisms other than *MTHFR* 677C>T had a significant impact on folate/Hcy phenotype. Here we report novel findings pertaining to RBC 5-MTHF: THF ratios and hence folate/Hcy phenotypes. The ratio between RBC 5-MTHF and THF seems to be a better indicator of the differences in RBC folate distribution than the absolute concentrations of either of the individual analytes. Caucasian *MTHFR* 1298C carriers had relatively low ratios of RBC 5-MTHF: THF. African American *MTRR* 66G carriers and *TYMS* 5' VNTR 2R carriers both had relatively high ratios of RBC 5-MTHF: THF. Both African American and Caucasian *RFC1* 80G carriers had relatively high ratios of RBC 5-MTHF: THF. *RFC1* is a transport protein that delivers 5-MTHF into cells. We can offer no biologically plausible explanation as to how this polymorphism would lead to different distributions of intracellular folates, and larger studies are needed to determine the validity of this observation.

In our study, tHcy levels in African American women were inversely related to RBC 5-MTHF levels, and tHcy levels in Caucasian women were inversely related to the RBC 5-MTHF: THF ratio. These findings are consistent with multiple reports, largely based on Caucasian populations, which have established that folate and Hcy concentrations have an inverse relationship (Kang et al., 1987; Selhub et al., 1993). Our observations suggest, however, that the above relationship may be more complex, in that the individual forms of folate and the proportional distribution of folates, exemplified by the RBC 5-MTHF: THF ratio, may have greater utility in predicting tHcy concentrations, at least in Caucasians.

Low levels of vitamin B₁₂ have been associated with high tHcy and low folate concentrations (Konstantinova et al., 2007; Thuesen et al., 2009). In our study, B₁₂ levels in both African Americans and Caucasians were directly related to RBC THF levels. B₁₂ is the cofactor for MTR, and our results indicate that B₁₂ levels facilitate the efficient generation of RBC THF from 5-MTHF by the action of MTR and MTRR.

Smoking has detrimental effects on folate/Hcy metabolism (Brown et al., 2004b; Gabriel et al., 2006; Stanislawski-Sachadyn et al., 2008b) and is associated with poor pregnancy outcome, such as orofacial clefts, preterm birth, and low birth weight infants (Meyer et al.,

1976; Wyszynski et al., 1997). In our study, African American smokers had lower RBC 5-MTHF, and this finding is consistent with other studies which found lower RBC folate in Caucasian smokers (Brown et al., 2004b; Gabriel et al., 2006). The higher prevalence of preterm birth and low birth weight infants in African American smokers than Caucasian smokers may be due, in part, to a more pronounced effect of smoking on folate/Hcy metabolism in the former population, but larger studies will be required to test this possibility.

In conclusion, the quantitatively precise methods that were used in this study to measure individual folate derivatives and other analytes have allowed the relationships between genetic/lifestyle factors and complex folate/Hcy phenotypes to be explored. Several of these relationships differed between African Americans and Caucasians, which may in part explain the differential risks for pregnancy-related pathologies in each of these groups. However, the number of subjects used in this study was relatively small, and these findings should be considered preliminary. Larger studies using similar analytical methods will be required to confirm these findings.

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Figure 1.

The folate/Hcy metabolic pathway. 5-MTHF, 5-methyltetrahydrofolate; 5,10-MTHF, 5,10-methenyltetrahydrofolate; CBS, cystathionine beta-synthase; DHF, dihydrofolate; DHFR, dihydrofolate reductase; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; Hcy, homocysteine; M, methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase 1958G>A; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; RFC1, reduced folate carrier 1; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SHMT1, serine hydroxymethyltransferase 1; THF, tetrahydrofolate; TYMS, thymidylate synthase.

Table 1

Subject characteristics and biochemical phenotypes (Mean±SD or n%).

	African American (N = 23)	Caucasian (N = 26)	Caucasian Subset (N=21) ¹
Subject Characteristics			
Age (years)	31.6 ± 6.0	33.3 ± 6.5	32.7 ± 6.1
Body mass index (kg/m ²)	28.3 ± 5.9	23.5 ± 3.4	23.6 ± 3.8
Smoking			
Yes	4 (17.4)	5 (19.2)	4 (19.0)
No	19 (82.7)	21 (80.8)	17 (81.0)
Alcohol use			
Yes	16 (69.6)	22 (84.6)	18 (85.7)
No	7 (30.4)	4 (15.4)	3 (14.3)
Vitamin use ²			
Yes	15 (65.2)	17 (65.4)	15 (71.4)
No	8 (34.8)	9 (34.6)	6 (28.6)
Biochemical Phenotypes			
Homocysteine (μmol/L)	8.9 ± 2.5	9.6 ± 2.7	8.9 ± 1.9
Total RBC folate (nmol/L) ³	939.1 ± 339.0	1186.0 ± 328.5	1165.3 ± 282.6
RBC 5-MTHF (nmol/L)	919.3 ± 334.1	1040.3 ± 333.0	1122.3 ± 278.8
RBC THF (nmol/L)	19.1 ± 9.1	118.2 ± 214.3	37.5 ± 31.2
RBC 5,10-MTHF			
Not Detectable	21 (91.3)	10 (38.5)	9 (42.9)
Detectable	2 (8.7)	16 (61.5)	12 (57.1)
Ratio RBC 5-MTHF: THF	51.5 ± 15.3	35.7 ± 23.6	42.9 ± 19.9
Plasma 5-MTHF (nmol/L)	33.5 ± 17.2	48.4 ± 20.5	50.2 ± 22.0
B ₁₂ (pmol/L)	542.4 ± 231.6	390.5 ± 176.6	393.0 ± 173.0

¹ After exclusion of *MTHFR* 677TT individuals.² Includes multivitamins, B vitamins, and folic acid.³ Total RBC folate = (RBC 5-MTHF) + (RBC THF) + (RBC 5, 10-MTHF)

Table 2

Genotype distributions (n%).

SNP (dbSNP rs no.)	Genotypes	African American (N = 23)	Caucasian (N = 26)	Caucasian Subset (N=21) ¹	P Value ²
<i>MTHFR</i> 677C>T	CC	16 (69.6)	8 (30.8)	8 (38.1)	0.009
rs1801133	CT	7 (30.4)	13 (50.0)	13 (61.9)	
	TT	0	5 (19.2)	0	
<i>MTHFR</i> 1298A>C	AA	13 (56.5)	14 (53.8)	9 (42.9)	0.61
rs1801131	AC	10 (43.5)	10 (38.5)	10 (47.6)	
	CC	0	2 (7.7)	2 (9.5)	
<i>MTR</i> 2756A>G	AA	10 (43.5)	15 (57.7)	14 (66.7)	0.17
rs1805087	AG	10 (43.5)	11 (42.3)	7 (33.3)	
	GG	3 (13.0)	0	0	
<i>MTRR</i> 66A>G	AA	13 (56.5)	6 (23.1)	5 (23.8)	0.039
rs1801394	AG	7 (30.4)	17 (65.4)	13 (61.9)	
	GG	3 (13.0)	3 (11.5)	3 (14.3)	
<i>CBS</i> 844ins68	WW	12 (52.2)	21 (80.8)	17 (80.9)	0.040
	WI	8 (34.8)	5 (19.2)	4 (19.1)	
	II	3 (13.0)	0	0	
<i>TYMS</i> 5' VNTR	2R/2R	3 (13.0)	2 (7.7)	1 (4.8)	0.14
	2R/3R	8 (34.8)	17 (65.4)	13 (61.9)	
	3R/3R	9 (39.1)	7 (26.9)	7 (33.3)	
	2R/4R	2 (8.7)	0	0	
	3R/4R	1 (4.4)	0	0	
<i>TYMS</i> 1494del6	ins/ins	2 (8.7)	9 (34.6)	7 (33.3)	0.006
rs16430	ins/del	11 (47.8)	15 (57.7)	12 (57.1)	
	del/del	10 (43.5)	2 (7.7)	2 (9.5)	
<i>DHFR</i> c.86+60_78	ins/ins	5 (21.7)	6 (23.1)	6 (28.6)	1
	ins/del	13 (56.5)	14 (53.8)	12 (57.1)	
	del/del	5 (21.7)	6 (23.1)	3 (14.3)	
<i>RFC1</i> 80A>G	AA	8 (34.8)	7 (26.9)	6 (28.6)	0.59

SNP (dbSNP rs no.)	Genotypes	African American (N = 23)	Caucasian (N = 26)	Caucasian Subset (N=21) ¹	P Value ²
rs1051266	AG	12 (52.2)	16 (61.5)	13 (61.9)	
	GG	3 (13.0)	3 (11.5)	2 (9.5)	
<i>MTHFD1</i> 1958G>A	GG	13 (56.5)	12 (46.2)	8 (38.1)	0.082
rs2236225	GA	8 (34.8)	5 (19.2)	3 (14.3)	
	AA	2 (8.7)	9 (34.6)	10 (47.6)	
<i>SHMT1</i> 1420C>T	CC	10 (43.5)	14 (53.8)	13 (61.9)	0.59
rs1979277	CT	8 (34.8)	9 (34.6)	8 (38.1)	
	TT	5 (21.7)	3 (11.5)	0	

¹ After exclusion of *MTHFR* 677TT individuals.

² P values by Fisher's exact test are comparison of genotype distributions in African Americans (n=23) and Caucasians (n=26).

Table 3

Proportion of variation (R^2) in biochemical variables explained by selected subject characteristics and biochemical phenotypes.

Dependent Variables	Subject Characteristics		African-American (N=23)		Caucasian Subset (N=21) ¹	
	Explanatory Variables	Intercept	Parameter Estimate (SE)	R^2 (P-value)	Parameter Estimate (SE)	R^2 (P-value)
Homocysteine ($\mu\text{mol/L}$)	Alcohol	Intercept-No Yes	7.5 (0.9) 2.0 (1.1)	0.15 (0.068)	8.3 (1.1) 0.8 (1.2)	0.02 (0.52)
Total RBC folate (nmol/L) ²	Smoking	Intercept-No Yes	1005.2 (71.7) -380.1 (171.9)	0.19 (0.038)	1200.9 (68.0) -189.7 (155.7)	0.07 (0.24)
RBC 5-MTHF (nmol/L)	Alcohol	Intercept-No Yes	1009.7 (130.6) -103.7 (156.5)	0.02 (0.51)	897.4 (153.7) 312.6 (166.0)	0.16 (0.075)
	Vitamin use	Intercept-No Yes	926.4 (90.0) 31.8 (152.6)	0.002 (0.84)	1072.1 (63.3) 326.5 (118.4)	0.29 (0.013)
	Smoking	Intercept-No Yes	984.6 (70.6) -375.4 (169.4)	0.19 (0.038)	1155.8 (67.1) -175.5 (153.8)	0.06 (0.27)
	Alcohol	Intercept-No Yes	987.5 (128.0) -98.0 (153.5)	0.02 (0.53)	875.0 (153.4) 288.5 (165.7)	0.14 (0.098)
RBC THF (nmol/L)	Vitamin use	Intercept-No Yes	910.5 (88.3) 25.2 (149.6)	0.001 (0.87)	1028.9 (62.0) 326.9 (116.1)	0.29 (0.011)
	RBC 5,10-MTHF	Intercept-ND ³ D	17.9 (1.8) 13.8 (6.2)	0.19 (0.037)	24.1 (9.9) 23.3 (13.0)	0.14 (0.089)
Ratio RBC 5-MTHF: THF	RBC 5,10-MTHF	Intercept-ND D	52.0 (3.4) -6.7 (11.5)	0.02 (0.57)	52.3 (6.2) -16.4 (8.2)	0.18 (0.059)
Plasma 5-MTHF (nmol/L)	Smoking	Intercept-No Yes	36.4 (3.8) -16.4 (9.0)	0.14 (0.083)	51.1 (5.5) -4.4 (12.5)	0.01 (0.73)
B ₁₂ (pmol/L)	Vitamin use	Intercept-No Yes	32.1 (4.5) 4.0 (7.7)	0.013 (0.60)	43.5 (5.1) 23.7 (9.5)	0.25 (0.022)
	Smoking	Intercept-No Yes	584.1 (49.8) -239.6 (119.5)	0.16 (0.058)	414.1 (41.6) -110.8 (95.3)	0.07 (0.26)

The parameter estimate for the intercept refers to the mean, and in the second line the parameter estimate is the difference between the two categories. Comparisons with P-values <0.10 in at least one group are listed.

¹ *MTHFR* 677TT individuals were removed for this analysis.

² Total RBC folate = (RBC 5-MTHF) + (RBC THF) + (RBC 5, 10-MTHF)

³ ND = Not Detectable, D = Detectable

Table 4

Proportion of variation (R^2) in biochemical variables explained by selected biochemical phenotypes.

Biochemical Phenotypes			African-American (N=23)		Caucasian (N=21) ^f	
Dependent Variables	Explanatory Variables	Parameter Estimate (SE)	R ² (P-value)	Parameter Estimate (SE)	R ² (P-value)	
Homocysteine (μmol/L)	Total RBC folate (nmol/L) ²	−0.003 (0.001)	0.23 (0.022)	−0.002 (0.001)	0.08 (0.22)	
	RBC 5-MTHF (nmol/L)	−0.004 (0.001)	0.23 (0.022)	−0.002 (0.001)	0.12 (0.12)	
	RBC THF (nmol/L)	−0.090 (0.056)	0.11 (0.12)	0.034 (0.012)	0.30 (0.010)	
	Ratio RBC 5-MTHF: THF	−0.006 (0.035)	0.002 (0.86)	−0.054 (0.018)	0.31 (0.009)	
Total RBC folate (nmol/L) ²	Plasma 5-MTHF (nmol/L)	0.065 (0.028)	0.21 (0.028)	−0.026 (0.019)	0.09 (0.19)	
	B ₁₂ (pmol/L)	−0.004 (0.002)	0.17 (0.049)	0.003 (0.002)	0.07 (0.24)	
	Plasma 5-MTHF (nmol/L)	16.12 (2.46)	0.67 (<0.0001)	6.63 (2.52)	0.27 (0.017)	
	B ₁₂ (pmol/L)	0.58 (0.29)	0.16 (0.063)	0.56 (0.35)	0.12 (0.13)	
RBC 5-MTHF (nmol/L)	RBC THF (nmol/L)	18.06 (6.95)	0.24 (0.017)	0.46 (2.01)	0.003 (0.82)	
RBC THF (nmol/L)	Plasma 5-MTHF (nmol/L)	16.05 (2.37)	0.69 (<0.0001)	6.65 (2.47)	0.28 (0.015)	
	B ₁₂ (pmol/L)	0.56 (0.29)	0.15 (0.069)	0.46 (0.35)	0.08 (0.21)	
	B ₁₂ (pmol/L)	0.018 (0.008)	0.21 (0.028)	0.09 (0.04)	0.26 (0.018)	
	Ratio RBC 5-MTHF: THF	Plasma 5-MTHF (nmol/L)	0.57 (0.15)	0.41 (0.001)	0.09 (0.21)	0.01 (0.68)

Parameter estimate refers to the change in units of the dependent variable for every 1 unit increase in the explanatory variable. Comparisons with P-values <0.10 in at least one group are listed.

^f *MTHFR* 677TT individuals were removed for this analysis.

² Total RBC folate = (RBC 5-MTHF) + (RBC THF) + (RBC 5, 10-MTHF)

Table 5Proportion of variation (R^2) in biochemical variables explained by selected genotypes in African Americans.

Dependent Variables	Genotype		African-American (N=23)	
			Parameter Estimate (SE)	R ² (P-value)
Homocysteine ($\mu\text{mol/L}$)	<i>MTR</i>	Intercept-AA	10.4 (0.7)	0.31
	2756A>G	G Carrier	-2.7 (0.9)	(0.006)
	<i>TYMS</i>	Intercept-del/del	10.2 (0.7)	0.22
	1494del6	Ins Carrier	-2.3 (0.9)	(0.023)
Total RBC folate (nmol/L) ¹	<i>MTR</i>	Intercept-AA	777.2 (99.2)	0.18
	2756A>G	G Carrier	286.4 (131.9)	(0.042)
RBC 5-MTHF (nmol/L)	<i>MTR</i>	Intercept-AA	760.7 (97.9)	0.18
	2756A>G	G Carrier	280.6 (130.2)	(0.043)
RBC THF (nmol/L)	<i>RFC1</i>	Intercept-AA	25.7 (2.8)	0.30
	80A>G	G Carrier	-10.2 (3.4)	(0.007)
Ratio RBC 5-MTHF: THF	<i>MTRR</i>	Intercept-AA	45.4 (3.9)	0.21
	66A>G	G Carrier	13.8 (5.9)	(0.028)
	<i>TYMS</i>	Intercept-3R/3R	41.3 (4.4)	0.32
	5' VNTR ²	2R Carrier	17.2 (5.9)	(0.010)
	<i>RFC1</i>	Intercept-AA	39.3 (4.5)	0.35
Plasma 5-MTHF (nmol/L)	80A>G	G Carrier	18.7 (5.5)	(0.003)
	<i>MTR</i>	Intercept-AA	25.5 (5.1)	0.17
	2756A>G	G Carrier	14.1 (6.8)	(0.049)
	<i>TYMS</i>	Intercept-3R/3R	26.1 (5.7)	0.16
	5' VNTR ²	2R Carrier	14.4 (7.7)	(0.077)

P-values <0.10 are listed.

¹Total RBC folate = (RBC 5-MTHF) + (RBC THF) + (RBC 5, 10-MTHF)²Genotypes containing 4R were removed from the analysis.

Table 6Proportion of variation (R^2) in biochemical variables explained by selected genotypes in Caucasians.

Dependent Variables	Genotype		Caucasian (N=26)	
			Parameter Estimate (SE)	R ² (P-value)
Homocysteine (μmol/L)	<i>MTHFR</i> 677C>T	Intercept-C Carrier TT	8.9 (0.5) 3.2 (1.2)	0.24 (0.012)
RBC 5-MTHF (nmol/L)	<i>MTHFR</i> 677C>T	Intercept-C Carrier TT	1122.3 (63.6) -426.3 (145.0)	0.26 (0.007)
RBC THF (nmol/L)	<i>MTHFR</i> 677C>T	Intercept-C Carrier TT	37.5 (29.4) 419.7 (67.1)	0.62 (<0.0001)
Ratio RBC 5-MTHF: THF	<i>MTHFR</i> 677C>T	Intercept-C Carrier TT	42.9 (4.0) -37.6 (9.2)	0.41 (0.0004)
Caucasian (N=21) ¹				
Homocysteine (μmol/L)	<i>MTHFR</i> 1298A>C	Intercept-AA C Carrier	7.9 (0.6) 1.8 (0.8)	0.22 (0.031)
	<i>MTR</i> 2756A>G	Intercept-AA AG	9.6 (0.5) -2.0 (0.8)	0.27 (0.017)
Total RBC folate (nmol/L) ²	<i>TYMS</i> 1494del6	Intercept-ins/ins Del Carrier	1019.9 (101.7) 218.1 (124.6)	0.14 (0.096)
RBC 5-MTHF (nmol/L)	<i>TYMS</i> 1494del6	Intercept-ins/ins Del Carrier	977.4 (100.2) 217.4 (122.7)	0.14 (0.092)
RBC THF (nmol/L)	<i>MTHFR</i> 1298A>C	Intercept-AA C Carrier	22.1 (9.6) 27.0 (12.7)	0.19 (0.046)
Ratio RBC 5-MTHF: THF	<i>MTHFR</i> 1298A>C	Intercept-AA C Carrier	55.6 (5.6) -22.1 (7.4)	0.32 (0.008)
	<i>MTR</i> 2756A>G	Intercept-AA AG	37.2 (5.0) 17.0 (8.6)	0.17 (0.061)
	<i>RFC1</i> 80A>G	Intercept-AA G Carrier	26.5 (7.0) 23.0 (8.3)	0.29 (0.012)
Plasma 5-MTHF (nmol/L)	<i>TYMS</i> 1494del6	Intercept-ins/ins Del Carrier	35.8 (7.5) 21.7 (9.2)	0.23 (0.030)

P-values <0.10 are listed.

¹ *MTHFR* 677TT individuals were removed for this analysis.² Total RBC folate = (RBC 5-MTH) + (RBC THF) + (RBC 5, 10-MT)

Table 7
Distribution of *MTHFR* 677 genotypes in Caucasians by detection of RBC 5,10-MTHF.

RBC 5,10-MTHF				
Genotype	Not Detectable (n)	Detectable (n)	P value	Detectable Mean (nmol/L) [Range] ^I
<i>MTHFR</i> ₆₇₇	CC	2	0.043	9.8 [6.8–12.7]
	CT	3		9.7 [4.9–20.0]
	TT	1		149.4 [74.6–224.7]

^I Mean of individuals having detectable levels.