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Folate and Homocysteine Phenotypes: Comparative Findings Using Research and Clinical Laboratory Data

Laura E. Mitchell^a, Megan Morales^b, Stefanie Khartulyari^c, Yuehua Huang^c, Kristen Murphy^b, Minghau Mei^a, Joan M. Von Feldt^b, Ian A. Blair^c, and Alexander S. Whitehead^{c,*}

^aInstitute of Biosciences and Technology, Texas A&M University System Health Science Center, Houston, TX

^bDivision of Rheumatology, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA

^cCenters for Pharmacogenetics, Excellence in Environmental Toxicology, and Cancer Pharmacology, Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA

Abstract

Objectives—A low folate/high homocysteine phenotype is associated with several pathologies, including spina bifida and cardiovascular disease. Folate and total homocysteine (tHcy) measurements are used clinically to assess risk and the need for folic acid supplementation and in research to investigate the metabolic basis of disease. Red blood cell (RBC) folate, the best indicator of long-term folate status, is usually measured as “total” folate. However, different folate derivatives support distinct biochemical functions, suggesting a need to develop more precise methods. This study was designed to evaluate a method based on stable isotope dilution liquid chromatography–multiple reaction monitoring/mass spectrometry (LC-MRM/MS).

Design and Methods—We used LC-MRM/MS to quantify the RBC folate derivatives 5-methyltetrahydrofolate (5-CH₃-THF), tetrahydrofolate (THF), and 5,10-methenyltetrahydrofolate (5,10-methenylTHF) in pre-menopausal women. The concentrations of each folate derivative was assessed for utility in predicting tHcy levels, and compared to folate and tHcy measurements derived by routine clinical laboratory methods.

Results—LC-MRM/MS was qualitatively and quantitatively superior to routine clinical laboratory methods for determining folate and tHcy concentrations. RBC 5-CH₃-THF had a reciprocal relationship with tHcy (p=0.0003), whereas RBC THF and RBC 5,10-methenylTHF had direct relationships (p=0.01, 0.04 respectively). In combination, these three variables accounted for 42% of the variation in tHcy.

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*Corresponding author: Department of Pharmacology, 153 Johnson Pavilion, 3620 Hamilton Walk, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6084, USA, aswhiteh@mail.med.upenn.edu, Tel: 215-898-2332, Fax: 215-573-9135.

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Conclusions—Robust methods for measuring RBC 5-CH₃-THF would improve the utility of folate/homocysteine phenotyping in patient management. The use of LC-MRM/MS would allow studies of hyperhomocysteinemia and diseases associated with a low folate/high homocysteine phenotype to be performed with less measurement error and greater statistical power to generate data with the potential to elucidate the etiologic mechanisms of complex diseases and traits.

Keywords

folate; homocysteine; hyperhomocysteinemia; disease risk; LC-MRM/MS

Introduction

Mild hyperhomocysteinemia, characterized by high (>13μmol/L) circulating concentrations [1] of the intermediate amino acid homocysteine (Hcy), has been associated with a wide range of human pathologies including cardiovascular disease [2], stroke [3], Alzheimer disease [4], cognitive impairment [5], inflammatory bowel disease [6], some cancers [7], and adverse reproductive outcomes, including birth defects such as spina bifida [8]. In a clinical setting, measurements of plasma total Hcy (tHcy) are made for a variety of purposes such as cardiovascular disease risk assessment and the management of patients taking anti-folate drugs. As elevated mild hyperhomocysteinemia is often underpinned by sub-optimal folate status [9,10], concurrent measurements of plasma folate and red blood cell (RBC) folate are often made.

Folate is a B vitamin that is centrally involved in one carbon metabolism (Figure 1). It is important for facilitating cellular methylation reactions involving substrates such as DNA, proteins and lipids, as well as xenobiotics and prescription medications, and for generating thymidylate and purines.¹¹ RBC folate concentrations are generally measured as “total folate” without distinguishing between the several forms of folate that are present. This potentially limits the predictive value of such measurements. The major circulating form of folate is 5-methyltetrahydrofolate (5-CH₃-THF). Intracellular 5-CH₃-THF, derived from 5,10-methylenetetrahydrofolate, is used to remethylate Hcy to methionine, which in turn is converted to the universal methyl donor S-adenosyl methionine. In the latter reaction 5-CH₃-THF is converted to tetrahydrofolate (THF). Alternatively, to facilitate nucleic acid synthesis, 5,10-methylenetetrahydrofolate is converted via 5,10-methenyltetrahydrofolate (5,10-methenylTHF) and formylated derivatives to THF. Thus, 5-CH₃-THF, THF and 5,10-methenylTHF represent distinct folate derivatives that play key roles within the methylation and nucleic acid synthesis compartments of folate/Hcy metabolism.

There is a well-established reciprocal relationship between folate and tHcy [9,10]. In addition to hyperhomocysteinemia, low folate status can lead to impaired methylating capacity, compromised nucleic acid synthesis, and increased glutathione production, all of which have pathogenic potential [11]. The importance of adequate folate status for preventing spina bifida is well established [12–14]. Indeed, to reduce the prevalence of birth defects such as spina bifida, folic acid fortification of milled grain products was mandated in the United States in 1998 [15].

Over the past two decades, many clinical studies have sought to characterize folate/Hcy metabolism in order to identify biochemical features that are associated with particular pathologies. In addition, studies of folate/Hcy phenotypes in healthy populations have been undertaken to examine the determinants of plasma and RBC folate levels and to assess the need for, or consequence of, folic acid supplementation and fortification. Many of these studies have relied on biochemical measurements that have been made in clinical laboratories using standard analytical methods and, for RBC folate, have considered only total folate levels.

The aim of the present study was to use a state of the art stable isotope dilution liquid chromatography–multiple reaction monitoring/mass spectrometry (LC-MRM/MS) method [16] for the quantitative analysis of three key forms of folate in RBCs (i.e. 5-CH₃-THF, THF and 5,10-methenylTHF), as well as RBC total folate as the sum of the above, and plasma 5-CH₃-THF, THF and tHcy in a defined segment of the healthy population. The concentrations of these variables in healthy, adult pre-menopausal females were determined, and results obtained using the above cutting edge techniques and standard clinical laboratory measurements are compared. The results have implications for the interpretation of data generated by clinical laboratories and for the design and conduct of future studies in which precise measurements of folate and Hcy are required.

Materials and Methods

Study Subjects

Pre-menopausal Caucasian and African American female subjects were recruited through advertisements from staff and students at the University of Pennsylvania School of Medicine. Exclusionary criteria were major medical conditions, especially autoimmune disease, use of anti-folate medications, and pregnancy. All subjects gave written informed consent. Subjects attended two study visits, approximately four weeks apart, at each of which a short questionnaire was administered and blood drawn. The study was approved by the Institutional Review Board of the University of Pennsylvania School of Medicine.

Laboratory Methods

Blood was drawn for two parallel sets of analyses. Separate aliquots from the same draw were directed to the clinical and research laboratories:

Clinical Laboratory—Routine measurements of biochemical variables of interest were carried out using standard assays by a hospital clinical laboratory. Specifically, assays used were AxSYM Homocysteine (Abbott Diagnostics) for total homocysteine (tHcy), Immulite 2500 Folic Acid (Siemens Medical Solutions Diagnostics) for serum folate, and Advia Centaur Folate (Siemens Medical Solutions Diagnostics) for RBC total folate. Complete blood count (CBC) determinations, including hematocrit, were performed using a Beckman-Coulter LH785/780 instrument.

Clinical laboratory values are as reported by the facility. tHcy concentrations are expressed as $\mu\text{mol/L}$. RBC total folate concentrations are expressed in ng/mL , rather than nmol/L , as

the analytical method used does not distinguish between the different constituent folate derivatives, for which the molecular masses differ slightly.

Research Laboratory—Stable isotope dilution LC-MRM/MS was used as previously described to measure tHcy [17], and plasma and RBC folate derivatives [16]. Blood for RBC folate measurements was lysed in 1% ascorbic acid at pH5 prior to analysis of 5-CH₃-THF and THF, and prior to acidification to pH1.5 with HCl for analysis of 5,10-methenylTHF as previously described in detail [16].

Similar to the clinical laboratory, the research laboratory values for tHcy concentrations are expressed as $\mu\text{mol/L}$. In contrast to the clinical laboratory, the research laboratory reported concentrations for each of three folate derivatives (5-CH₃-THF, THF, and 5,10-methenylTHF), as well as for RBC total folate as the sum of the three derivatives, in nmol/L .

Statistical Methods

Descriptive analyses were undertaken to characterize the subjects enrolled in the study. Continuous variables were summarized using means, standard deviations, medians and ranges, and discrete variables were summarized using counts and proportions. Serum folate values, as measured by the clinical laboratory, were dichotomized ($\leq 15 \text{ ng/mL}$ versus $>15 \text{ ng/mL}$) because this laboratory reported all values above 15 ng/mL as $>15 \text{ ng/mL}$. The research laboratory measures of RBC 5,10-methenylTHF and plasma THF were considered as both continuous and discrete (not detectable versus $>0 \text{ nmol/L}$) variables because of the high proportion of non-detectable values for each (63.3% and 20.4%, respectively). Differences between measures of the same variable obtained at the first and second visits were summarized using the absolute difference and its standard error.

Agreement between the clinical and research laboratory measurements of tHcy, and relative bias were assessed as described by Bland and Altman [18]. In addition, the distributions of subjects across three clinically relevant subgroups defined by clinical or research laboratory tHcy values: $>13 \mu\text{mol/L}$ indicating hyperhomocysteinemia and the need to consider high dose folic acid therapy; $10\text{--}13 \mu\text{mol/L}$ indicating the need to retest; and $<10 \mu\text{mol/L}$ indicating values in the desirable range, were compared. Because the clinical and research laboratories used different units of measurement for RBC folate, agreement between these two measures could not be directly assessed. Consequently, the mean of the absolute difference between the values obtained for RBC total folate at visits 1 and 2 was expressed as a proportion of the mean value at visit 1, and the resulting proportions were compared for the clinical and research laboratory measures.

The strength of the relationship between the clinical laboratory measures of serum and RBC total folate, and the research laboratory measures of plasma folate, RBC total folate, and RBC folate derivatives were assessed using the Pearson correlation coefficient. In addition, linear regression analyses were used to assess the strength of the relationship between tHcy and both serum/plasma and RBC total folate levels, as measured by the clinical and research laboratories, and the strength of the relationship between tHcy and levels of the three individual folate derivatives, as measured by the research laboratory. All statistical analyses were conducted using SAS version 9.13 (SAS Institute Inc., Cary, NC).

Results

A total of 53 subjects were consented into the study; however, after recognition of exclusionary conditions and medication use, 49 subjects (age 22–49 years) were enrolled: 26 and 23 self-identified as Caucasian and African American respectively. The second study visits ranged from 24 to 39 days (mean 32.6 days) after the first visit.

The mean value of the absolute difference between the tHcy values obtained at Visit 1 and Visit 2 was small for both the clinical and research laboratory measures (Table 1), indicating that this aspect of phenotype is relatively stable over a period of approximately one month. However, the standard deviation of this mean was considerably larger for the clinical laboratory (SD=2.2) than for the research laboratory (SD=1.3), indicating that the research laboratory values provide a more precise measure of tHcy.

At both Visit 1 and Visit 2, mean tHcy concentrations obtained by the research laboratory were approximately 20% lower than those reported by the clinical laboratory (Visit 1: 9.2 vs 11.1; Visit 2: 9.0 vs 11.5). To explore this disparity between the tHcy values measured by the clinical and research laboratory methods, the difference between the clinical and research laboratory values at Visit 1 was plotted against the mean of these two values for each subject (Figure 2). It is clear from this plot that the values obtained by the clinical laboratory tend to be greater than those obtained for the same subject by the research laboratory (mean difference = 1.88 μ mol/L, standard deviation = 1.74). The limits of agreement (mean difference $\pm$ 2SD) indicate that the majority (~95%) of the clinical laboratory values will fall between 1.60 μ mol/L below and 5.4 μ mol/L above the value obtained in the research laboratory. However, the maximum observed difference between the clinical and laboratory values of tHcy was 15.1 μ mol/L.

Further comparison of the clinical and research laboratory tHcy measures, using clinically relevant cut-points (i.e. <10 μ mol/L, 10–13 μ mol/L, >13 μ mol/L), indicated that the two laboratories provided the same classification for only 50% of the 98 study visits (i.e. 49 Visit 1 plus 49 Visit 2), and that there was an approximately two-fold difference in the number of visits at which subjects would be considered normal and require no follow-up or intervention (Table 2). Specifically, based on the clinical laboratory results (at Visit 1 or Visit 2) approximately 30% of subjects would be classified as being in the normal range (tHcy <10 μ mol/L), whereas approximately 65% of subjects would be classified as such based on the research laboratory results.

The mean values of the absolute difference between the RBC total folate values at Visit 1 and Visit 2 (Table 1), like those for tHcy, were relatively small for both the clinical and research laboratory measures. However, for the clinical laboratory measures, the mean of the absolute difference was 19% of the visit 1 mean value, whereas for the research laboratory this proportion was only 10%, suggesting that the research laboratory provides a more precise measure of RBC total folate than does the clinical laboratory.

The mean of the absolute difference between the research laboratory measures of RBC 5-CH₃-THF at Visit 1 and Visit 2 was also relatively small (10% of the Visit 1 mean), indicating that RBC 5-CH₃-THF is relatively stable over a period of approximately one

month. As expected, this difference was considerably larger for the research laboratory measures of plasma RBC 5-CH₃-THF (26% of the Visit 1 mean). RBC THF also appeared to be relatively stable across this period (i.e. mean of absolute difference, 12% of Visit 1 mean). However, RBC 5,10-methenylTHF appeared to be more variable (i.e. mean of absolute difference, 35% of Visit 1 mean).

Serum folate levels, as determined by the clinical laboratory, were significantly correlated ($r=0.60$) with the plasma 5-CH₃-THF levels obtained by the research laboratory (Table 3). In addition, clinical laboratory RBC total folate levels were significantly correlated ($r=0.73$) with the research laboratory RBC total folate, as well as with each of the individual constituent folate derivatives (5-CH₃-THF, THF and 5,10-methenylTHF). Among the derivatives, clinical laboratory RBC total folate was most highly correlated with 5-CH₃-THF ($r=0.55$). However, it is of note that all correlation coefficients were substantially below 1.0, indicating that RBC total folate measures obtained by the clinical laboratory provide relatively poor proxy measures for the individual RBC folate derivatives.

Linear regression analyses of the relationship between tHcy and the various folate measures were undertaken separately in data from the clinical and research laboratories. Using data generated by the clinical laboratory, neither RBC total folate nor serum folate concentration was significantly related to tHcy concentration (Table 4), and the proportion of variation in tHcy levels accounted for by these variables was small (1% and 3%). In contrast, several of the RBC and plasma folate derivatives (measured by the research laboratory) were significantly related to the research laboratory's measures of tHcy. Most notable is the highly significant ($p=0.0003$) inverse relationship between RBC 5-CH₃-THF and tHcy, the significant ($p=0.01$) direct relationship between RBC THF and tHcy, and the significant ($p=0.04$) direct relationship between RBC 5,10-methenylTHF and tHcy. Individually, these variables accounted for 25%, 13% and 9% of the variation in tHcy, respectively. It is of note that the relationship between tHcy and RBC total folate, which is the sum of the above RBC folate derivatives, was of only borderline significance ($p=0.07$) and this measure accounted for only 7% of the variation in tHcy. This is not unexpected as this composite measure includes components that differ in the direction of their relationships with tHcy. Plasma 5-CH₃-THF was also significantly ($p=0.02$) and inversely related to tHcy, but this association, which accounted for 10% of tHcy variation, was not as strong as that with RBC 5-CH₃-THF. In contrast to RBC THF, plasma THF was inversely related to tHcy concentrations, but this association was of only borderline significance ($p=0.08$). When the research laboratory values of RBC 5-CH₃-THF, THF, and 5,10-methenylTHF were included in a regression model for tHcy, in combination they accounted for 42% of the variation in tHcy and each of these variables was significantly related to tHcy ($p=0.0003$, $p=0.006$ and $p=0.002$, respectively).

Discussion

Individuals with a low folate/high Hcy phenotype are considered to be at increased risk of several human pathologies [19]. In particular, maternal low folate status before and very early in the first trimester of pregnancy is a risk factor for spina bifida in offspring [8], and elevated tHcy is a risk marker for a range of atherothrombotic diseases [20]. There is an

inverse relationship between folate and tHcy concentrations [10,11], and several supplementation studies have shown that daily doses of as little as 200µg folic acid or more effect a “normalizing” reduction in tHcy concentrations as well as a *de facto* improvement in folate status [21,22]. In addition, diets that are rich in sources of natural folate, such as the Mediterranean diet, may be effective in lowering tHcy concentrations, particularly in those with a genetic predisposition to hyperhomocysteinemia [23].

Patients with tHcy concentrations above 13µmol/L are generally classified as having mild hyperhomocysteinemia, considered to be at elevated cardiovascular disease risk, and in many clinical practices would be prescribed a daily supplement containing 1mg or more folic acid. A subsequent tHcy reading that remained above 13µmol/L might trigger an increase in the amount of folic acid prescribed to 2mg or even 5mg per day. Commonly prescribed branded formulations are available in doses that reflect the above levels; for example, Folgard OS and Folgard RX tablets contain 1.1mg folic acid and are often dispensed as a 30-day supply containing 60 tablets. tHcy measurements between 10µmol/L and 13µmol/L might warrant retesting and in the event of a second test yielding a tHcy value above 13µmol/L, the above decisions regarding the prescribing of folic acid supplements would come into play.

Our data suggest that tHcy measurements made by some clinical laboratories might be considerably different from those obtained using quantitatively precise LC-MRM/MS methods. In our study, the former exceeded the latter by approximately 2µmol/L on average; however, there was considerable intra-individual variation in the absolute difference between the two analytic measurements, with single visit clinical laboratory values for some subjects being as much as 15.1µmol/L higher than the research laboratory values. Consequently, some individuals with tHcy levels less than 10µmol/L by LC-MRM/MS have clinical laboratory measurements above 13µmol/L (5 of 98 assay pairs) or between 10µmol/L and 13µmol/L (32 of 98 assay pairs), which in a clinical setting might respectively trigger intervention with high dose folic acid by prescription or retesting. Furthermore, there were nine tHcy values at 10–13µmol/L by LCMRM/MS that were >13µmol/L by clinical laboratory assay that might trigger folic acid prescription rather than retesting.

The above findings are from a comparison of LC-MRM/MS measurements of tHcy with those obtained in a single clinical laboratory. However, if confirmed in other comparative studies, these findings would suggest that a substantial number of patients for whom tHcy measurements are routinely obtained might be inappropriately classified as hyperhomocysteinemic. Such misclassification could divert practitioners from a full clinical evaluation and treatment of other cardiovascular risk factors and might lead, in some individuals, to the generation of inaccurate cardiovascular risk profiles that could result in the initiation of unwarranted long-term remediation with high, or even very high, dose folic acid supplements. The issue of whether high dose folic acid supplements themselves constitute a health risk with respect to cancer has recently emerged [24]. While a determination of the validity of such concerns over possible adverse consequences will require further large studies, it seems prudent to limit the number of individuals exposed to daily doses of folic acid that are between five and twenty-five times those (i.e. 200µg per day) that are known to be sufficient to resolve mild hyperhomocysteinemia in the majority

of the population, and that are multiples higher than the recommended dietary reference intake [21,22].

The above considerations suggest that LC-MRM/MS could be used in conjunction with the methods for measuring folates that are currently used by clinical laboratories to determine clinically relevant cut-points, thereby facilitating standard criteria for interventions using folic acid supplements.

RBC total folate levels are considered to be better indicators of long-term folate status, and to better reflect tissue levels, than serum/plasma folate concentrations, which are more transient. The data presented here indicate that the best specific measure for evaluating the *reciprocal* relationship between folate and tHcy concentrations is RBC 5-CH₃-THF. Indeed, RBC THF and 5,10-methenylTHF concentrations were both *positively* correlated with tHcy concentrations. This clearly establishes the utility of developing robust methods appropriate for clinical laboratories that could be used to accurately assay RBC 5-CH₃-THF when folate status is being evaluated in the context of tHcy concentrations and other folate-dependent phenotypic variables.

Finally, the data presented here support the use of cutting edge, quantitatively precise assays for measuring both folates and tHcy in human studies designed to explore the complex inter-relationships between components of folate/Hcy metabolism *per se*, and in the context of hyperhomocysteinemia and pathologies in which a low folate/high Hcy phenotype has been causally implicated. The precision of these assays will result in less measurement error and, hence, improved power relative to studies that employ quantitatively less precise assays. Where possible future studies in which folate and tHcy concentrations are an integral part should assess RBC 5-CH₃-THF (and possibly other folate derivatives), rather than RBC total folate, using quantitatively precise methods such as LC-MRM/MS. Such studies will permit more reliable conclusions to be drawn regarding folate/Hcy metabolism *per se*, and its contribution to disease etiology.

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References

1. Jacques PF, Selhub J, Bostom AG, Wilson PW, Rosenberg IH. The effect of folic acid fortification on plasma folate and total homocysteine concentration. *N Engl J Med.* 1999; 340:1449–1454. [PubMed: 10320382]
2. Refsum H, Ueland M, Nygård O, Vollset SE. Homocysteine and cardiovascular disease. *Ann Rev Medicine.* 1998; 49:31–62.
3. Furie KL, Kelly PJ. Homocysteine and stroke. *Semin Neurol.* 2006; 26:24–32. [PubMed: 16479441]
4. Seshadri S. Elevated plasma homocysteine levels: risk factor or risk marker for the development of dementia and Alzheimer's disease? *J Alzheimers Disease.* 2006; 9:393–398. [PubMed: 16917147]

5. Durga J, van Boxtel MP, Schouten EG, Kok FJ, Jolles J, Katan MB, Verhoef P. Effect of 3-year folic acid supplementation on cognitive function in older adults in the FAVIT trial: a randomized, double blind, controlled trial. *Lancet*. 2007; 369:208–216. [PubMed: 17240287]
6. Mahmud N, Molloy AM, McPartlin J, Corbally R, Whitehead AS, Scott JM, Weir DG. Increased prevalence of methylenetetrahydrofolate reductase C677T variant in patients with inflammatory bowel disease, and its clinical implications. *Gut*. 1999; 45:389–394. [PubMed: 10446107]
7. Powers HJ. Interaction among folate, riboflavin, genotype, and cancer, with reference to colorectal and cervical cancer. *J Nutr*. 2005; 135:2960S–2966S. [PubMed: 16317155]
8. Mitchell LE, Adzick NS, Melchionne J, Pasquariello PS, Sutton LN, Whitehead AS. Spina bifida. *Lancet*. 2004; 364:1885–1895. [PubMed: 15555669]
9. Jacques PF, Bostom AG, Williams RR, Ellison RC, Eckfeldt JH, Rosenberg IH, Selhub J, Rozen R. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. *Circulation*. 1996; 93:7–9. [PubMed: 8616944]
10. Harmon DL, Woodside JV, Yarnell JWG, McMaster D, Young IS, McCrum EE, Gey KF, Whitehead AS, Evans AE. The common thermolabile variant of methylene- tetrahydrofolate reductase is a major determinant of mild hyperhomocysteinaemia. *Q J Med*. 1996; 89:571–577.
11. Stover PJ. Physiology of folate and B12 in health and disease. *Nutr Res*. 2004; 62:3–12.
12. MRC Vitamin Study Research Group: Prevention of neural tube defects: results of the Medical Research Council vitamin study. *Lancet*. 1991; 338:131–137. [PubMed: 1677062]
13. Czeizel A, Dudas I. Prevention of the first occurrence of neural tube defects by periconceptional vitamin supplementation. *N Engl J Med*. 1992; 327:1832–1835. [PubMed: 1307234]
14. Barry RJ, Erickson JD, Li S, Moore CA, Wang H, Molinare J, Zhao P, Wang LY, Gindler J, Hong SX, Correa A. Prevention of neural tube defects with folic acid in China. China-U.S. Collaborative Project for Neural Tube Defects. *N Engl J Med*. 1999; 341:1485–1490. [PubMed: 10559448]
15. Food standards: amendments of the standards of identity for enriched grain products to require addition of folic acid. *Fed Regist*. 1996; 61:8781–8797.
16. Huang Y, Khartulyari S, Morales S, Stanislawski-Sachadyn A, Von Feldt JM, Whitehead AS, Blair IA. Quantification of key red blood cell folates from subjects with defined MTHFR 677C>T genotypes using stable isotope dilution liquid chromatography/mass spectrometry. *Rapid Commun Mass Spectrom*. 2008; 22:1–10. [PubMed: 18041795]
17. Huang Y, Lu Z-Y, Brown KS, Whitehead AS, Blair IA. Quantification of intracellular homocysteine by stable isotope dilution liquid chromatography/tandem mass spectrometry. *Biomed Chromatogr*. 2007; 21:107–112. [PubMed: 17121413]
18. Bland JM, Altman DG. Statistical methods for assessing agreement between methods of clinical measurement. *Lancet*. 1986; i:307–310. [PubMed: 2868172]
19. Lucock MD. Synergy of genes and nutrients: the case of homocysteine. *Curr Opin Clin Nutr Metab Care*. 2006; 9:748–756. [PubMed: 17053430]
20. Refsum H, Ueland M, Nygård O, Vollset SE. Homocysteine and cardiovascular disease. *Ann Rev Medicine*. 1998; 49:31–62.
21. Guttormsen AB, Ueland PM, Nesthus I, Nygard O, Schneede J, Vollset SE, Refsum H. Determinants and vitamin responsiveness of intermediate hyperhomocysteinemia (≥ 40 micromol/liter). The Hordaland Homocysteine Study. *J Clin Invest*. 1996; 98:2174–2183. [PubMed: 8903338]
22. Ward M, McNulty H, McPartlin J, Strain JJ, Weir DG, Scott JM. Plasma homocysteine, a risk factor for cardiovascular disease, is lowered by physiological doses of folic acid. *QJM*. 1997; 90:519–524. [PubMed: 9327030]
23. Dedoussis GV, Panagiotakos DB, Chrysoshoou C, Pitsavos C, Zampelas A, Choumerianou D, Stefanidis C. Effect of interaction between adherence to a Mediterranean diet and the methylenetetrahydrofolate reductase 677C>T mutation on homocysteine concentrations in healthy adults: the ATTICA study. *Am J Clin Nutr*. 2004; 80:849–854. [PubMed: 15447889]
23. Cole BF, Baron JA, Sandler RS, Haile RW, Ahnen DJ, Bresalier RS, McKeown-Eyssen G, Summers RW, Rothstein RI, Burke CA, Snover DC, Church TR, Allen JI, Robertson DJ, Beck GJ, Bond JH, Byers T, Mandel JS, Mott LA, Pearson LH, Barry EL, Rees JR, Marcon N, Saibil F,

Ueland PM, Greenberg ER. Folic acid for the prevention of colorectal adenomas: a randomized clinical trial. *JAMA*. 2007; 297:2351–2359. [PubMed: 17551129]

Figure 1.

Folate/Hcy metabolism. Abbreviations: AdoHcyH, *S*-adenosylhomocysteine hydrolase; CBS = cystathionine β -synthase; DHF, dihydrofolate; DHFR = dihydrofolate reductase; dUMP = 2'-deoxyuridine monophosphate; FTHF, formyltetrahydrofolate; FTHFL = formate tetrahydrofolate ligase; GR = glutathione reductase; GSH = glutathione; Hcy = homocysteine; MAT = methionine adenosyltransferase; METHF, methylenetetrahydrofolate; MT, methyltransferase; MTHF, methenyltetrahydrofolate; MTHFC = methylenetetrahydrofolate cyclohydrolase; MTHFD = methylenetetrahydrofolate dehydrogenase; MTHFR = methylenetetrahydrofolate reductase; MTR = methionine synthase; MTRR = methionine synthase reductase; SAH = *S*-adenosyl-homocysteine; SAM = *S*-adenosylmethionine; SHMT = serine hydroxymethyltransferase; THF, tetrahydrofolate; TMP = thymidine monophosphate; TYMS = thymidylate synthase; Vit = vitamin.

Figure 2.

Agreement between clinical and research laboratory measures of tHcy: difference versus average of tHcy values measured by the clinical and research laboratories.

Table 1

Summary of the biochemical phenotypes observed at Visit 1 and Visit 2.

Variables	Mean ± SD (median, range) or N (%)		Mean of Absolute Difference ± SD (%) ^a
	Visit 1	Visit 2	
Clinical Laboratory Phenotypes			
Homocysteine (μmol/L)	11.1 ± 2.6 (11.3, 6.4–18.1)	11.5 ± 3.3 (11.3, 6.6–23.8)	2.3 ± 2.2 (20.8)
RBC total folate (ng/mL)	639.8 ± 152.0 (639.0, 397.0–1032.0)	695.8 ± 192.5 (677.5, 383.0–1224.0)	122.9 ± 103.2 (19.2)
Serum folate			
15 ng/mL	19 (38.8)	20 (40.8)	--
> 15 ng/mL	30 (61.2)	29 (59.2)	--
Research Laboratory Phenotypes			
Homocysteine (μmol/L)	9.2 ± 2.6 (9.0, 4.5–16.9)	9.0 ± 2.6 (8.7, 5.5–19.1)	1.3 ± 1.2 (13.6)
RBC total folate (nmol/L) ^b	1069.1 ± 354.0 (1063.4, 507.6–2077.7)	1052.7 ± 356.7 (1088.5, 422.5–2190.6)	106.3 ± 88.9 (10.0)
RBC 5-CH ₃ -THF (nmol/L)	983.5 ± 335.6 (963.1, 202.3–1661.1)	968.9 ± 326.2 (1016.1, 149.9–1636.2)	103.5 ± 86.8 (10.5)
RBC THF (nmol/L)	70.7 ± 163.1 (21.3, 0.0–889.9)	68.9 ± 159.8 (21.0, 0–864.9)	8.7 ± 14.8 (12.3)
RBC 5,10-methenylTHF (nmol/L)	14.9 ± 45.3 (0.0, 0.0–224.7)	14.9 ± 37.5 (0, 0–198.5)	5.2 ± 12.7 (35.2)
RBC 5,10-methenylTHF ND ^c	31 (63.3)	25 (51.0)	--
>0 nmol/L	18 (36.7)	24 (49.0)	--
Plasma 5-CH ₃ -THF (nmol/L)	41.4 ± 20.3 (39.4, 6.2–91.3)	42.1 ± 19.2 (41.4, 8.0–81.2)	10.7 ± 9.1 (25.8)
Plasma THF (nmol/L)	0.7 ± 0.4 (0.7, 0.0–1.5)	0.6 ± 0.5 (0.7, 0–1.5)	0.3 ± 0.3 (44.3)
Plasma THF ND ^c	10 (20.4)	16 (32.7)	--
>0 nmol/L	39 (79.6)	33 (67.3)	--

^a% = mean of absolute difference/Visit 1 mean^bRBC total folate = (RBC 5-CH₃-THF) + (RBC THF) + (RBC 5, 10-methenylTHF)^cND = non-detectable

Table 2
Comparison of homocysteine levels as measured by the clinical and research laboratories.

Research Laboratory	Clinical Laboratory						
	Visit 1			Visit 2			
	< 10 µmol/L	10 – 13 µmol/L	> 13 µmol/L	Total	< 10 µmol/L	10 – 13 µmol/L	> 13 µmol/L
< 10 µmol/L	13	18	0	31 (.63)	15	14	5
10 – 13 µmol/L	2	8	4	14 (.29)	1	6	5
> 13 µmol/L	0	0	4	4 (.08)	0	0	3
Total	15 (.31)	26 (.53)	8 (.16)	49	16 (.33)	20 (.41)	13 (.26)
							49

Table 3

Correlations between serum and RBC folate measurements from the clinical laboratory and selected folate derivative measurements from the research laboratory.

Clinical Laboratory	Research Laboratory	Correlation Coefficient (P-value)
Serum folate (ng/mL) ^a	Plasma 5-CH ₃ -THF (nmol/L)	0.60 (<0.01)
Serum folate (ng/mL) ^a	Plasma THF (nmol/L)	0.55 (<0.01)
RBC total folate (mg/mL)	RBC total folate (nmol/L) ^b	0.73 (<0.01)
RBC folate (mg/mL)	RBC 5-CH ₃ -THF (nmol/L)	0.55 (<0.01)
RBC folate (mg/mL)	RBC THF (nmol/L)	0.36 (0.01)
RBC folate (mg/mL)	RBC 5,10 methenylTHF (nmol/L)	0.38 (0.01)

^a Serum folate: 15 ng/mL versus >15 ng/mL

^b RBC folate = (RBC 5-CH₃-THF) + (RBC THF) + (RBC 5,10-methenylTHF)

Table 4

Summary of linear regression analyses of homocysteine measurements from the clinical and research laboratories.

Dependent variables	Predictors	Parameter estimate (SE)	R ² (p-value)
Clinical Laboratory			
Homocysteine (μmol/L)	RBC total folate (ng/mL) ^a	−0.002 (0.002)	0.01 (0.43)
Homocysteine (μmol/L)	Serum folate (ng/mL) ^{ba}	−0.84 (0.76)	0.03 (0.28)
Research Laboratory			
Homocysteine (μmol/L)	RBC total folate (nmol/L) ^b	−0.002 (0.001)	0.07 (0.07)
Homocysteine (μmol/L)	RBC 5-CH ₃ -THF (nmol/L)	−0.004 (0.001)	0.25 (0.0003)
Homocysteine (μmol/L)	RBC THF (nmol/L)	0.006 (0.002)	0.13 (0.01)
Homocysteine (μmol/L)	RBC 5,10-methenylTHF(nmol/L)	0.02 (0.01)	0.09 (0.04)
Homocysteine (μmol/L)	Plasma 5-CH ₃ -THF (nmol/L)	−0.04 (0.02)	0.10 (0.02)
Homocysteine (μmol/L)	Plasma THF (nmol/L)	−1.64 (0.93)	0.06 (0.08)

^aRBC total folate = (RBC 5-CH₃-THF) + (RBC THF) + (RBC 5,10-methenylTHF)

^{ab}Serum folate: 15mg/mL versus >15mg/mL

^bRBC total folate = (RBC 5-CH₃-THF) + (RBC THF) + (RBC 5,10-methenylTHF)