Influence of 5,10-Methylenetetrahydrofolate Reductase Polymorphism on Whole-Blood Folate Concentrations Measured by LC-MS/MS, Microbiologic Assay, and Bio-Rad Radioassay

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BACKGROUND: The 5,10-methylenetetrahydrofolate reductase (NADPH) (MTHFR) C677T polymorphism may affect whole-blood folate pattern measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and total folate measured by LC-MS/MS, microbiologic assay, and Bio-Rad radioassay (BR).

METHODS: We analyzed 171 whole blood hemolysates from 2 blood banks for folate pattern and total folate concentrations using these 3 methods and determined MTHFR genotype.

RESULTS: The median (range) total folate concentration by LC-MS/MS was higher in the US set [378 (228–820) nmol/L; n = 96] than in the European set [250 (122–582) nmol/L; n = 75]. The whole-blood folate pattern [median (range)] was similar for individuals with C/C (n = 73) and C/T (n = 66) genotype: 88% (71%–91%) and 86% (50%–91%), respectively, for 5-methyltetrahydrofolic acid (5CH3THF) vs 12% (9%–29%) and 14% (9%–51%) for forms other than 5-methyltetrahydrofolic acid (non-5CH3THF). Individuals with T/T (n = 32) genotype had 58% (22%–87%) 5CH3THF vs 42% (13%–78%) non-5CH3THF. Compared with microbiologic assay results, LC-MS/MS (r = 0.94) and BR (r = 0.87) results were significantly lower (−10% and −45%, respectively); however, these differences were concentration dependent and also genotype dependent for the BR assay (−48% for C/C+C/T and −31% for T/T). The microbiologic assay completely recovered [mean (SD)] folates added to a whole blood hemolysate, except for tetrahydrofolic acid (THF) [46.4% (8.1%)]. The BR assay underrecovered 5CH3THF [51% (4.1%)] and 5-formyltetrahydrofolic acid [18% (0.1%)], and overrecovered THF [152% (19%)].

CONCLUSION: MTHFR C677T polymorphism influences the folate pattern in whole blood. The agreement between total folate by LC-MS/MS and microbiologic assay, independent of the MTHFR genotype, allows the use of one regression equation. Because BR results are genotype dependent, different regression equations should be used.

Brief Communications

Erythrocyte or whole-blood folate concentrations represent whole-body folate stores and are used to assess folate status. We and others have shown that the 5,10-methylenetetrahydrofolate reductase (NADPH) (MTHFR) 677C>T mutation results in accumulation of reduced folates other than 5-methyltetrahydrofolic acid (5CH3THF), which is the predominant folate form usually found in mature erythrocytes (1–5). Under conditions of impaired folate status, the T/T genotype has been associated with increased risk of neural tube defects, colorectal neoplasias, and possibly cardiovascular disease (6). An earlier report raised concerns that the variation of erythrocyte folate in MTHFR polymorphism is related to the assay used (7). The Bio-Rad QuantaPhase II radioassay (BR), used since 1991 to monitor the folate status of the US population through the National Health and Nutrition Examination Survey (NHANES), was discontinued in 2007; 2 new methods are now employed, liquid chromatography-tandem mass spectrometry (LC-MS/MS) (3, 8, 9) and the microbiologic assay (10, 11). We used 3 different assays to investigate the influence of the MTHFR polymorphism on whole-blood folate pattern and response, and we assessed whether regression equations could be derived for future time trend analyses of NHANES.

To cover a wide range of folate concentrations, we purchased EDTA anticoagulated whole blood samples from 2 blood banks. These specimens included 96 and 75 samples from US and European blood donors, respectively, collected in spring 2006. We also purchased nonanticoagulated whole blood samples from the US blood bank to prepare matching serum samples. Whole blood samples were stored at −70 °C before hemolysis with 1% (wt/vol) L-ascorbic acid, pH 2.7 (1:11 dilution). The same hemolysate, incubated at 37°C for 4 h to ensure complete folate deconjugation (8) and then frozen at −70 °C, was used for LC-MS/MS folate pattern analysis and for total folate (TFOL) analysis by microbiologic assay. A hemolysate without incubation was used for the BR; the manufacturer states that no incubation is needed if the he-

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1 Nonstandard abbreviations: LC-MS/MS, liquid chromatography-tandem mass spectrometry; BR, Bio-Rad radioassay; 5CH3THF, 5-methyltetrahydrofolate acid; THF, tetrahydrofolic acid; TFOL, total folate; 5CHOTHF, 5-formyltetrahydrofolic acid; 5,10CH2THF, 5,10-methylenetetrahydrofolic acid; SSR, sum of squared residuals; PRESS, predicted residual sum of squares; FA, folic acid.
molsate is frozen and assayed later. MTHFR C677T (rs1801133) polymorphism was genotyped using the MGB Eclipse™ probe system (Nanogen) (12).

We analyzed the data with SAS (version 9; SAS Institute) and Analyze-it for Microsoft Excel software. The effect of genotype (C/C, C/T, and T/T) and data set (US and Europe) on TFOL concentrations was studied by 2-factor ANOVA for each method separately. For descriptive statistics, each data set was analyzed separately. In addition to individual folate species, we also calculated the ratio of 5CH₃THF to TFOL and of reduced folates other than 5CH₃THF [non-5CH₃THF; sum of 5-formyltetrahydrofolic acid (5CHOTHF), 5,10-methenyltetrahydrofolic acid (5,10CH=THF), and tetrahydrofolic acid (THF)] to TFOL, as described previously (1–5). To compare the methods, we used the sum of folate species determined by LC-MS/MS and TFOL determined by microbiologic assay or BR. Method regression equations are presented only for the combined set because we found no differences between the 2 data sets other than the concentration ranges. Methods were compared by least-squares regression, after log-transformation of the data, to account for nongaussian distribution. As in our method comparison paper for serum folate (13), we developed multiple linear regression models that used a dummy binary variable (0, 1), IND, to account for intercept differences, and an interaction variable (IND × log₁₀ microbiologic assay) to account for differences in slope over 2 discrete concentration intervals (cutoff of 370 nmol/L for microbiologic assay representing the median concentration). The fit of the models was evaluated by comparing the sum of squared residuals (SSR) to the predicted residual sum of squares (PRESS).

We tested the recoveries of the microbiologic assay and BR methods by adding each of the 5 folate calibrators (Merck Eprova) at 10 nmol/L [in 1% (wt/vol) L-ascorbic acid, pH 2.7, for microbiologic assay and in protein diluent for BR] to a whole-blood hemolysate pool.

The concentrations of folate species in whole blood were higher in the US than in the European sample set, as was the median (range) TFOL concentration by LC-MS/MS [378 (228–820) nmol/L, US set (n = 96); 250 (122–582) nmol/L, European set (n = 75)] (Table 1). The relative whole-blood folate pattern [median (range)] was similar for individuals with C/C (n = 73) and C/T (n = 66) genotype, regardless of the sample set: 88% (71%–91%) and 86% (50%–91%), respectively, for 5CH₃THF vs 12% (9%–29%) and 14% (9%–51%) for non-5CH₃THF (see also Fig. 1 in Data Supplement). Individuals with T/T (n = 32) genotype displayed 58% (22%–87%) 5CH₃THF vs 42% (13%–78%) non-5CH₃THF forms, with 5,10CH=THF being the largest contributor to the non-5CH₃THF portion. We did not find differences in serum folate pattern or TFOL by genotype (see Table 1 in the Data Supplement).

We found a significant effect of data set (P < 0.001) on TFOL concentrations determined by LC-MS/MS or microbiologic assay, but no effect of genotype and no genotype × data set interaction [see Fig. 2 in the Data Supplement for scatter plots by genotype (panel A) and by data set (panel C)]. The LC-MS/MS and microbiologic assays showed a correlation of r = 0.94, but LC-MS/MS produced slightly lower values, resulting in a significant negative concentration-dependent difference of −10% (95% CI, −12% to −8%; 2 SD limits of agreement, −32% to 20% based on log₁₀-transformed Bland Altman analysis). The multiple linear regression equation was as follows:

\[
\log_{10} \text{LC-MS/MS} = 0.2959 + (0.8697 \times \log_{10} \text{microbiologic assay}) - (0.1582 \times \text{IND}) + (0.0534 \times \text{IND} \times \log_{10} \text{microbiologic assay}),
\]

with IND=0 for microbiologic assay results ≤370 nmol/L and IND=1 for microbiologic assay results >370 nmol/L (Fig. 1A). The SSR (0.5406) agreed with the PRESS (0.5679).

The BR assay produced lower results than the other 2 assays. Because of a significant genotype × data set interaction (P = 0.0041), the 2-factor ANOVA for this assay was followed by 1-factor ANOVA, looking at the effect of genotype in each data set separately. In both data sets, genotype had a significant effect on TFOL concentrations determined by BR (US, P = 0.0424, C/T different from T/T; Europe, P = 0.0081, C/C different from T/T) [see Fig. 2 in the Data Supplement for scatter plots by genotype (panel B) and by data set (panel D)]. We therefore analyzed the data separately for C/C + C/T and T/T genotype. We also present data based on all genotypes. The BR and microbiologic assays showed a correlation of r = 0.87 for all genotypes, r = 0.94 for C/C + C/T, and r = 0.88 for T/T, but BR produced lower values, resulting in a significant negative concentration-dependent difference: −45% for all genotypes (95% CI, −46% to −43%; 2 SD limits of agreement, −62% to −20% based on log₁₀-transformed Bland Altman analysis); −48% for C/C + C/T (95% CI, −46% to −43%; 2 SD limits of agreement, −62% to −20%); −31% for T/T (95% CI, −35% to −26%; 2 SD limits of agreement, −52% to 0%). The multiple linear regression equations were as follows:

For all genotypes,

\[
\log_{10} \text{BR} = 0.3479 + (0.7606 \times \log_{10} \text{microbiologic assay}) - (0.0875 \times \text{IND}) + (0.0357 \times \text{IND} \times \log_{10} \text{microbiologic assay}),
\]

SSR = 0.8953, PRESS = 0.9343 (Fig. 1B).
Table 1. Descriptive statistics for whole-blood folate concentrations measured by LC-MS/MS, microbiologic assay, and BR methods.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>5CH\textsubscript{3}THF</th>
<th>5CHOTHF</th>
<th>THF</th>
<th>5,10CH\textsubscript{2}THF</th>
<th>LC-MS/MS</th>
<th>Microbiologic assay</th>
<th>BR</th>
</tr>
</thead>
<tbody>
<tr>
<td>US sample set</td>
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<td></td>
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<tr>
<td>All study participants</td>
<td>96</td>
<td></td>
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<td></td>
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<tr>
<td>Concentration, nmol/L</td>
<td>304 (94.7–703)</td>
<td>41.4 (22.7–93.9)</td>
<td>0 (0–142)</td>
<td>10.1 (0–212)</td>
<td>378 (228–820)</td>
<td>449 (240–1086)</td>
<td>230 (116–505)</td>
<td></td>
</tr>
<tr>
<td>Ratio to TFOL, %</td>
<td>86 (22–92)\textsuperscript{b}</td>
<td>14 (8.5–79)\textsuperscript{c}</td>
<td></td>
<td></td>
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<tr>
<td>MTHFR 677 C/C</td>
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<tr>
<td>Concentration, nmol/L</td>
<td>317 (180–600)</td>
<td>38.6 (22.7–73.8)</td>
<td>0 (0–23.5)</td>
<td>8.79 (0–23.5)</td>
<td>365 (243–698)</td>
<td>383 (263–915)</td>
<td>202 (121–382)</td>
<td></td>
</tr>
<tr>
<td>Ratio to TFOL, %</td>
<td>87 (71–91)</td>
<td>13 (8.5–29)</td>
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<td>MTHFR 677 C/T</td>
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<tr>
<td>Concentration, nmol/L</td>
<td>321 (175–703)</td>
<td>42.5 (25.7–93.9)</td>
<td>0 (0–142)</td>
<td>10.8 (0–49.8)</td>
<td>383 (251–820)</td>
<td>448 (272–1086)</td>
<td>220 (116–437)</td>
<td></td>
</tr>
<tr>
<td>Ratio to TFOL, %</td>
<td>86 (49–89)</td>
<td>14 (11–51)</td>
<td></td>
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<td>MTHFR 677 T/T</td>
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<td></td>
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<tr>
<td>Concentration, nmol/L</td>
<td>248 (94.7–414)</td>
<td>49.8 (27.1–88.7)</td>
<td>0 (0–68.7)</td>
<td>73.5 (9.00–212)</td>
<td>368 (228–598)</td>
<td>412 (240–956)</td>
<td>265 (171–505)</td>
<td></td>
</tr>
<tr>
<td>Ratio to TFOL, %</td>
<td>63 (22–87)</td>
<td>37 (13–78)</td>
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European sample set

| All study participants | 75 | | | | | | |
| Concentration, nmol/L | 207 (30.2–462) | 29.2 (13.1–68.9) | 0 (0–23.5) | 9.65 (0–167) | 250 (122–582) | 242 (120–671) | 155 (66.6–434) | |
| Ratio to TFOL, % | 85 (23–91) | 15 (8.8–77) | | | | | | |
| MTHFR 677 C/C | | | | | | | |
| Concentration, nmol/L | 239 (120–462) | 30.8 (16.5–64.4) | 0 (0–16.7) | 7.68 (0–16.5) | 275 (136–543) | 242 (158–522) | 155 (76.7–289) | |
| Ratio to TFOL, % | 88 (80–91) | 13 (8.8–20) | | | | | | |
| MTHFR 677 C/T | | | | | | | |
| Concentration, nmol/L | 205 (109–385) | 28.8 (13.0–46.2) | 0 (0–17.8) | 8.16 (0–18.4) | 238 (123–441) | 236 (120–461) | 141 (66.6–229) | |
| Ratio to TFOL, % | 86 (78–91) | 14 (9.0–22) | | | | | | |
| MTHFR 677 T/T | | | | | | | |
| Concentration, nmol/L | 150 (30.2–334) | 34.9 (18.5–68.9) | 0 (0–23.5) | 69.3 (10.3–167) | 238 (122–582) | 270 (138–671) | 203 (98.3–434) | |
| Ratio to TFOL, % | 57 (23–83) | 43 (17–77) | | | | | | |

Data are median (range).\textsuperscript{a} Zero is recorded instead of the limit-of-detection (LOD) values, which are as follows: THF, 0.29 nmol/L hemolysate; 5,10CH\textsubscript{2}THF, 0.55 nmol/L hemolysate.\textsuperscript{b} Ratio between 5CH\textsubscript{3}THF and TFOL by LC-MS/MS.\textsuperscript{c} Ratio between non-5CH\textsubscript{3}THF (5CHOTHF + THF + 5,10CH\textsubscript{2}THF) and TFOL by LC-MS/MS.

For C/C+C/T,
\[
\log_{10} \text{BR} = 0.3040 + (0.7695 \times \log_{10} \text{microbiologic assay}) - (0.1389 \times \text{IND}) + (0.0551 \times \text{IND} \times \log_{10} \text{microbiologic assay}),
\]
\[
\text{SSR} = 0.3879, \text{PRESS} = 0.4120 \text{ (Fig. 1C)}.
\]

For T/T,
\[
\log_{10} \text{BR} = -0.3160 + (1.0837 \times \log_{10} \text{microbiologic assay}) - (0.6236 \times \text{IND}) + (0.1932 \times \text{IND} \times \log_{10} \text{microbiologic assay}),
\]
\[
\text{SSR} = 0.1457, \text{PRESS} = 0.1862 \text{ (Fig. 1D)}.
\]

Our recovery experiments with whole blood samples measured by microbiologic assay showed satisfactory recovery [mean (SD), n = 3 d] for 5CH\textsubscript{3} THF [97% (11%)], 5CHOTHF [124% (7%)], and 5,10CH\textsubscript{2}THF [107% (13%)] and underrecovery for THF [46.4% (8%)]. These results compared well with...
Multiple linear regression model, all MTHFR C677T genotypes:
\[ \log_{10} \text{LC-MS/MS} = 0.2959 + (0.8697 \times \log_{10} \text{MA}) - (0.1582 \times \text{IND}) + (0.0534 \times \text{IND} \times \log_{10} \text{MA}), \]
IND = 0 for MA ≤370 nmol/L, and IND = 1 for MA >370 nmol/L.
\[ r = 0.94; \text{SSR} = 0.5406; \text{PRESS} = 0.5679. \]

Multiple linear regression model, all MTHFR C677T genotypes:
\[ \log_{10} \text{BR} = 0.3479 + (0.7606 \times \log_{10} \text{MA}) - (0.0875 \times \text{IND}) + (0.0357 \times \text{IND} \times \log_{10} \text{MA}), \]
IND = 0 for MA ≤370 nmol/L, and IND = 1 for MA >370 nmol/L.
\[ r = 0.87; \text{SSR} = 0.8953; \text{PRESS} = 0.9343. \]

Multiple linear regression model, MTHFR C/C + C/T genotypes:
\[ \log_{10} \text{BR} = 0.3040 + (0.7695 \times \log_{10} \text{MA}) - (0.1389 \times \text{IND}) + (0.0551 \times \text{IND} \times \log_{10} \text{MA}), \]
IND = 0 for MA ≤370 nmol/L, and IND = 1 for MA >370 nmol/L.
\[ r = 0.94; \text{SSR} = 0.3879; \text{PRESS} = 0.4120. \]

Multiple linear regression model, MTHFR T/T genotypes:
\[ \log_{10} \text{BR} = 0.3160 + (1.0837 \times \log_{10} \text{MA}) - (0.6236 \times \text{IND}) + (0.1932 \times \text{IND} \times \log_{10} \text{MA}), \]
IND = 0 for MA ≤370 nmol/L, and IND = 1 for MA >370 nmol/L.
\[ r = 0.88; \text{SSR} = 0.1457; \text{PRESS} = 0.1862. \]

Fig. 1. Least-squares regression plots for TFOL measured in whole blood in the combined sample set by 3 methods: (A) LC-MS/MS vs microbiologic assay for all genotypes, (B) BR vs microbiologic assay for all genotypes, (C) BR vs microbiologic assay for C/C+C/T genotypes, and (D) BR vs microbiologic assay for T/T genotype.
The microbiologic assay is used as the reference point \( n = 171 \) (A) and \( n = 139 \) (C), and \( n = 32 \) (D).
our previous report of recoveries in serum samples (13). The BR showed satisfactory recovery (n = 2 days) for 5,10CH = THF [115% (10%)], but underrecovered 5CH3THF [51% (4%)] and 5CHOTHF [18% (0.1%)]) and overrecovered THF [152% (19%)]. The underrecovery of 5CH3THF and 5CHOTHF, also seen in serum samples (13), could be a primary cause for the lower results obtained by BR. The shift in folate pattern in favor of non-5CH3THF in the T/T genotype appears to account for the higher responses of the BR for this genotype.

The current study investigated the influence of MTHFR C677T polymorphism on whole blood folate pattern and response by 3 different assays. As reported previously (1–5), our results confirm that individuals with the T/T genotype showed an accumulation of non-5CH3THF forms (approximately 30%–40%) in their whole blood. The novelty of this study is that it analyzed whole blood from a larger number of individuals with T/T vs C/C genotype, only when folate status was excluding patients taking vitamins (2, 13). Thus, our finding indicates that the folate status of the individuals in the European sample set might be adequate. We confirmed previous findings of high interindividual variability in non-5CH3THF accumulation in individuals with the T/T genotype (from <20% to 80% of TFOL) (4–5). We observed less interindividual variability in individuals with the C/C or C/T genotype for the European set than for the US set. Owing to the lack of a genotype effect for the LC-MS/MS and microbiologic assay, we were able to provide one regression equation that was applicable to a wide concentration range and that covers populations with and without folate fortification. However, we had to use separate equations for the BR assay depending on genotype. The equation for all genotypes will provide only a rough estimate in situations in which genotype information is not available.

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