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Red blood cell folate vitamer distribution in healthy subjects is determined by the methylenetetrahydrofolate reductase C677T polymorphism and by the total folate status☆

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Abstract

Background: Red blood cells (RBCs) represent a storage pool for folate. In contrast to plasma, RBC folate can appear in different biochemical isoforms. So far, only the methylenetetrahydrofolate reductase (MTHFR) 677 TT genotype has been identified as a determinant of RBC folate vitamer distribution.

Objective: The purpose of this study is to identify clinical and biochemical determinants of RBC folate vitamer distribution in healthy subjects.

Design: In an observational study, 109 subjects, aged 18 to 65 years, were studied. Red blood cell folate vitamers were analyzed using a liquid chromatography–tandem mass spectrometry method. Other variables recorded included vitamin B_2, B_6 and B_{12} status, homocysteine, plasma and RBC *S*-adenosylhomocysteine and *S*-adenosylmethionine, renal function and the MTHFR C677T polymorphism.

Results: The MTHFR C677T genotype was the dominant determinant of nonmethylfolate accumulation. The median (range) nonmethylfolate/total folate ratio was 0.58% (0–12.2%) in the MTHFR CC group ($n=55$), 0.99% (0–14.3%) in the CT group ($n=39$) and 30.3% (5.7–73.3%) in the TT genotype group ($n=15$), $P<.001$. The 95th percentile for the nonmethylfolate/total folate ratio was 2.8% for the CC group, 9.1% for the CT group and 73.3% for the TT group. In the CC and CT genotype subjects, the T-allele and total folate status were positively and independently correlated with nonmethylfolate accumulation, but the degree of nonmethylfolate accumulation in these subjects was usually minor compared with those with the TT genotype. None of the other studied variables was associated with nonmethylfolate accumulation.

Conclusions: The MTHFR C677T genotype is the dominant determinant of nonmethylfolate accumulation in RBCs. In addition, high total folate status may contribute to minor to moderate nonmethylfolate accumulation in MTHFR CC and CT subjects.

Keywords: Folate; B vitamins; Methylenetetrahydrofolate reductase (MTHFR); Mass spectrometry; Red blood cell

1. Introduction

Folate status is related to a variety of diseases, including neural tube defects [1], neurological and cognitive disturbances [2], cancer [3] and cardiovascular disease [4,5]. The diversity of postulated mechanisms linking folate status to disease is closely related to the different biochemical functions of folate. Folate's main role is to enable enzymes to pass on one-carbon groups to a variety of target molecules. This way, folate plays a role in the regulation of homocysteine homeostasis, in providing methyl groups to support transmethylation reactions and in supplying one-carbon units to DNA/RNA synthesis (Fig. 1). To fulfill these diverse metabolic tasks, folate appears in different chemical forms, or "folate vitamers." The differences between these

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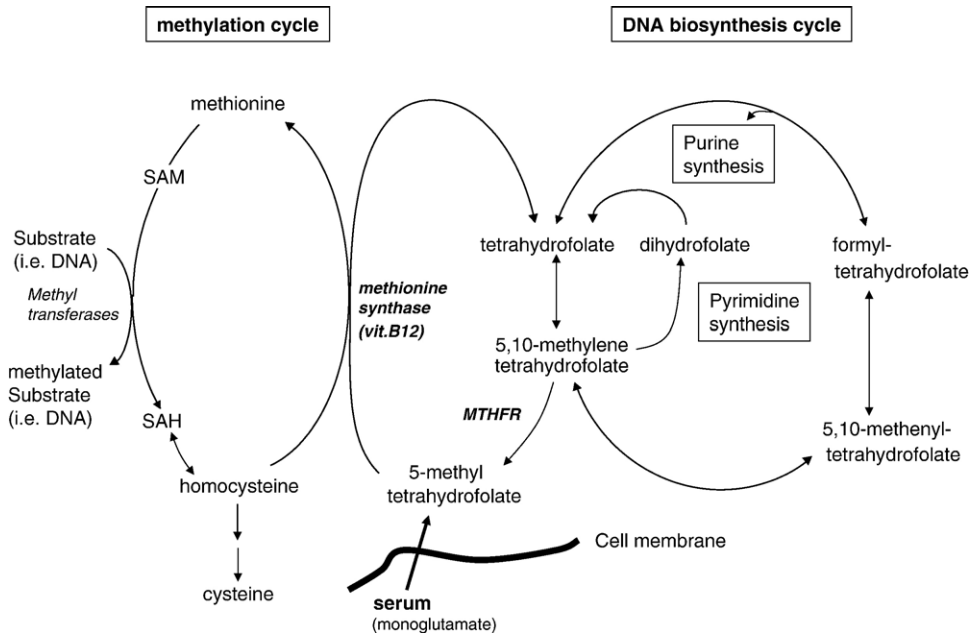

Fig. 1. Overview of cellular folate metabolism.

vitamers are in the oxidation state of the pteridine ring, the one-carbon substitution at the N5 and N10 position and the number of conjugated glutamic acid moieties attached to the folate molecule.

To gain more understanding of the pathogenesis of diseases related to folate (deficiency), it is necessary to study the distribution of folate vitamers in biological samples. Human plasma is unsuitable for this purpose because folate in plasma occurs almost exclusively as 5-methyltetrahydrofolate (5MTHF) [6]. Hence, measurement of intracellular folate vitamer distribution is required.

In the past, folate vitamer analysis has been hampered by technical difficulties, relating to incomplete extraction of folate from cells, chemical instability of several folate vitamers and the presence of different polyglutamate chain lengths attached to folate molecules. Partly because of these analytical issues, knowledge of normal intracellular folate vitamer distribution is limited. Because the red blood cell (RBC) represent a storage pool for folate, several recent studies have focused on RBC folate vitamer distribution. A few studies have shown that homozygosity for the methylenetetrahydrofolate reductase (MTHFR) 677 C>T mutation results in accumulation of reduced folates other than 5MTHF, which is the folate usually found in mature RBCs [6–9]. Other potential determinants of RBC folate distribution have, however, not been systematically studied.

We recently developed a highly sensitive and precise analytical method for measuring RBC folate vitamer distribution [10]. In this article, we provide the results of this method in a cohort of healthy adult volunteers, over a wide age range (18–65 years). The purpose is to study potential determinants of RBC folate vitamer distribution. In particular, we are interested in whether folate vitamer distribution is influenced by total folate status, by other B vitamins or by *S*-adenosylmethionine and *S*-adenosylhomocysteine, all factors influencing cellular folate metabolism.

2. Methods

2.1. Study participants

Healthy volunteers, aged 18 to 65 years, were recruited from hospital staff by advertisement. Subjects were excluded if one of the following was present: pregnancy, use of any type of (multi-)vitamin tablet in the preceding year, use of any drug other than oral contraceptives or signs of active infectious or inflammatory disease. Written informed consent was obtained from all participants. The hospital local ethics committee approved the study protocol.

2.2. Analytical methods

After an overnight fast, blood was drawn between 8:00 and 10:00 a.m., and was immediately placed on ice. Blood with no anticoagulant was used for the analysis of serum vitamin B_{12} (2 ml), serum pyridoxal-5-phosphate (1 ml) and serum total folate levels (2 ml). Blood with heparin added as anticoagulant was used for the analysis of whole-blood vitamin B_2 levels (2 ml). Blood with EDTA added as a coagulant was used for the analysis of hematocrit (1 ml), whole-blood SAM/SAH (1 ml), plasma SAM/SAH (1 ml), whole-blood total folate (1 ml), whole-blood folate fractionation (0.1 ml) and DNA extraction (0.2 ml). Plasma was isolated from RBCs by centrifugation for 10 min at 3000 rpm at 4°C within 15 min after collection. Hematocrit and serum creatinine were determined immediately. The remain-

Table 1
Clinical and biochemical characteristics of the study population

	Mean±S.D.*	Range
Age (years)	36±11.3	18–62
Gender (male/female)	52/57	
Body mass index (kg/m^2)	24.6±3.5	18.8–33.2
Smoking (*n*)	13	
Serum creatinine (μmol/L)	89±11	67–115
Plasma folate (nmol/L)	8.8*	3.1–32.4
Serum B_{12} (pmol/L)	236*	46–561
Plasma pyridoxal-5-phosphate (nmol/L)	41*	11–181
Flavin adenine dinucleotide (μmol/L)	0.31±0.04	0.20–0.36
Plasma homocysteine (μmol/L)	9.7*	4.3–52.9
Plasma *S*-adenosylhomocysteine (nmol/L)	13.1*	7.4–32.9
RBC *S*-adenosylhomocysteine (nmol/L)	215±37.3	130–318
Plasma *S*-adenosylmethionine (nmol/L)	84.9±15.5	57.1–141.0
RBC *S*-adenosylmethionine (nmol/L)	3394±714	1685–5777
MTHFR C677T genotype		
CC	55	
CT	39	
TT	15	
MTHFR A1298C genotype		
AA	57	
AC	39	
CC	13	

* Median.

der of the samples were stored at −80°C until further analysis.

Plasma folate and serum vitamin B_{12} concentrations were measured by competitive immunoassay (Architect; Abbott Laboratories, Abbott Park, IL). Inter- and intraassay CVs were 4% and 6%, respectively, for folate and 3.5% and 6%, respectively, for vitamin B_{12}. Previously, we have found that at least 97% of plasma folate consists of 5MTHF, the remainder being reduced nonmethylfolates (unpublished data). Normal values (locally validated) were >150 pmol/L for vitamin B_{12} and >6.5 nmol/L for folate. Pyridoxal-5-phosphate levels (normal value, >13 nmol/L) were measured in serum by HPLC (interassay CV, 3%) [11]. Vitamin B_2 status was determined by the analysis of flavin adenine dinucleotide in whole blood using HPLC (normal values, 0.20–0.36 μmol/L) [12]. Plasma total homocysteine concentration (normal value, <18 μmol/L) was measured by automated fluorescence polarization immunoassay (IMx analyser, Abbott, interassay coefficient of variation 4%). For SAM and SAH measurements in whole blood, samples were deproteinized immediately after collection by the addition of 1 ml of 5% perchloric acid to 1 ml of whole blood (100 μl of the supernatant was used for the analysis). For SAM and SAH measurements in plasma, samples were deproteinized immediately after centrifugation by the addition of 0.625 ml of 10% perchloric acid solution to 1 ml plasma. (Five hundred microliters of the supernatant was used for the analysis.) Tandem mass spectrometry was used to determine SAM and SAH in plasma and whole blood (intraassay CV, 4% for both determinations; interassay CV, 8% and 6%, respectively) [13]. Erythrocyte concentrations of SAM and SAH were calculated as

$$\frac{\textit{whole blood concentration} - \textit{plasma concentration}\ (1 - \textit{hematocrit})}{\textit{hematocrit}}$$

Hematocrit was determined by electronic measurement of packed cell volume.

DNA was isolated from EDTA blood by a QIAamp DNA blood kit (Qiagen, Hilden, Germany). The C677T polymorphism of MTHFR was assessed by polymerase chain reaction, as previously described [14]. In addition, the A1298C mutation in MTHFR was analyzed by polymerase chain reaction using molecular beacons [15].

2.2.1. Red blood cell folate analysis

Details of the LC-MS/MS method have been described elsewhere [10]. In short, whole-blood samples were collected in EDTA vacutainers and hemolyzed by freeze thawing. The lysates were diluted in ascorbic acid solutions and placed in a 37°C water bath for 90 min, allowing for deconjugation by naturally occurring plasma conjugase. After addition of an extraction buffer, proteins were denatured in a boiling water bath for 20 min. Samples were then purified using folate binding protein affinity columns, concentrated by SPE and evaporation, and subsequently stored at −20°C until analysis. After reconstitution in mobile phase and LC separation on a C_{18} column within 6 min, folates were detected using positive electrospray ionization MS/MS under multiple reaction monitoring conditions. Because all one-carbon-substituted nonmethylfolate vitamers (particularly 10-formyltetrahydrofolate and 5,10-methylenetetrahydrofolate) largely converted to 5,10-methenyltetrahydrofolate under the acidic analytical conditions of our method, the output is expressed as 5MTHF and a pooled result for nonmethyltetrahydrofolates, as is done by others [6,16]. Limits of quantification (*S*/*N*>10) for

Table 2
Red blood cell folate vitamer concentrations

Genotype (*n*)	RBC non-MTHFR (nmol/L), median (range)	RBC MTHFR (nmol/L), median (range)	RBC total folate (nmol/L), median (range)	Non-MTHFR/total folate ratio (%), median (range)
All subjects (109)	4.1 (0–786.2)	427.3 (92.5–1085.8)	440.9 (170.3–1164.4)	0.9 (0–73.3)
MTHFR 677 CC (55)	3.5 (0–67.0)	450.7 (263.8–1085.8)	459.1 (263.8–1086.9)	0.58 (0–12.2)
MTHFR 677 CT (39)	3.9 (0–166.9)	403.3 (205.5–997.5)	412.0 (210.1–1164.4)	0.99 (0–14.3)
MTHFR 677 TT (15)	138.7 (13.8–786.2)	273.8 (92.5–514.3)	436.9 (170.3–1072.1)	30.3 (5.7–73.3)
P value (Kruskal–Wallis)	<.001	<.001	.32	<.001

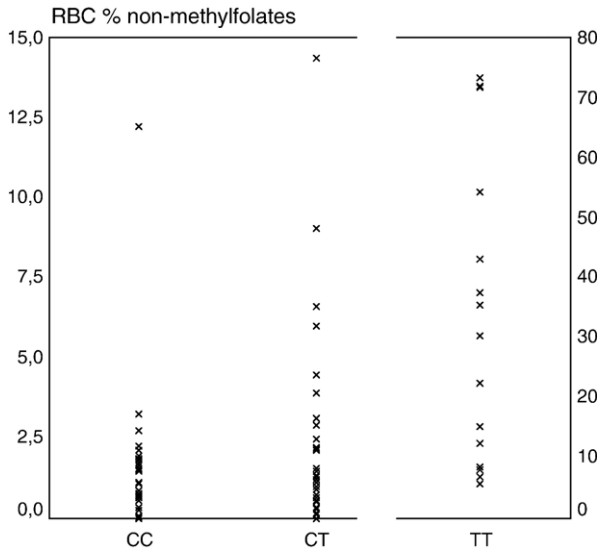

Fig. 2. Red blood cell nonmethylfolate expressed as % of RBC total folate in three MTHFR C677T genotype groups. Note different scales of *y*-axis for the CC and CT genotype (left axis) and the TT genotype (right axis).

5MTHF and for non-methylTHF were 0.4 nmol/L. Intraassay and interassay CVs for 5-methylTHF were 1.2% and 2.8%, respectively. Intraassay and interassay CVs for non-methylTHF as a group were 1.6% and 1.5%, respectively. Red blood cell levels were obtained by dividing whole-blood levels by hematocrit, except for 5MTHF, for which RBC concentrations were calculated taking into account plasma folate. Total RBC folate levels were obtained by adding up the 5MTHF and nonmethylfolate concentrations. Red blood cell total folate levels were also measured directly by competitive immunoassay (Architect, Abbott Laboratories).

2.2.2. Statistical analysis

Data are represented as mean (S.D.) or as median (range). Routine bivariate tests were performed as indicated in the Results section. For correlation analyses, data were analyzed as continuous variables unless stated otherwise. Nonlinear associations were checked by analyzing potential determinants of RBC nonmethylfolate accumulation divided in tertiles. Multiple groups were compared by one-way analysis of variance or, in case of a nonnormal distribution, Kruskal–Wallis analysis.

3. Results

In total, 109 individuals were studied. Their characteristics are listed in Table 1. Some individuals were found to have mild to moderate B-vitamin deficiencies, but subjects were not excluded for this reason.

Red blood cell folate vitamer concentrations are listed in Table 2. In the whole group, there was marked variability and positive skewness in the nonmethylfolate/total folate ratio. Analysis of the folate vitamers in the three different MTHFR C677T genotype groups shows that the TT genotype accounted for most of the cases with a relatively high nonmethylfolate/total folate ratio (Table 2 and Fig. 2). The MTHFR 677 CT genotype appeared to represent at most a very mild intermediate phenotype with respect to the nonmethylfolate/total folate ratio, the difference with the MTHFR 677 CC group just failing to reach statistical significance ($P=.06$). The 95th percentile for the nonmethylfolate/total folate ratio was 2.8% for the CC group, 9.1% for the CT group and 73.3% for the TT group.

Because the MTHFR 677 TT genotype was the most dominant determinant of nonmethylfolate accumulation, analyses aimed at identifying additional determinants of the nonmethylfolate/total folate ratio were done separately in the CC and CT genotype group on the one hand and the TT group on the other. The following variables were considered as potential determinants of the RBC nonmethylfolate/total folate ratio: age, gender, total folate status, vitamin B_{12}, pyridoxal-5-phosphate, flavin adenine dinucleotide, plasma and RBC levels of *S*-adenosylmethionine and *S*-adenosylhomocysteine, and the MTHFR A1298C genotype. Of these, only RBC total folate turned out to be positively correlated to the RBC nonmethylfolate/total folate ratio, but only in the MTHFR CC and CT group (Fig. 3).

Multiple regression analysis showed that, within the combined MTHFR 667 CC and CT genotype group, the T allele (standardized $\beta=.25$; $P=.01$) and RBC total folate status (standardized $\beta=.34$; $P=.001$) were independently associated with RBC nonmethylfolate accumulation. Replacing RBC total folate measured by LC-MS/MS in the regression model by total RBC folate measured by competitive immunoassay, as well as exclusion of subjects with nondetectable RBC nonmethylfolates, yielded identical results (data not shown). Serum total folate measured by chemiluminescence did not significantly correlate with RBC

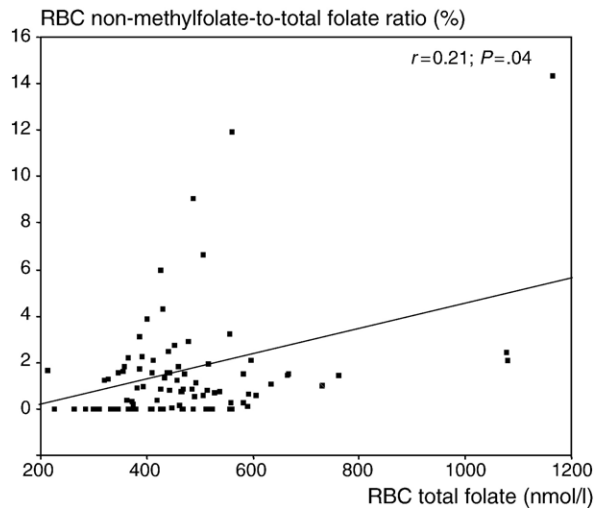

Fig. 3. Scatter plot of the association between RBC total folate and the nonmethylfolate/total folate ratio in subjects with the MTHFR 677 CC or CT genotype. Significance of the correlation is maintained after exclusion of the three patients with relatively high folate status of >800 nmol/L.

nonmethylfolate accumulation (data not shown). Within the MTHFR 677 TT genotype group, none of the studied variables (partly) explained the marked variation in the RBC nonmethylfolate/total folate ratio.

4. Discussion

Our study confirms previous findings that marked (up to 75% of total folate) nonmethylfolate accumulation in RBCs is mainly found in subjects with the MTHFR 677 TT genotype. Individuals with the MTHFR 677 CT genotype have slightly higher values for the RBC nonmethylfolate/total folate ratio than those with the CC genotype. Reference values for the RBC nonmethylfolate/total folate ratio, as indicated by the 95th percentile levels, are clearly highest in the TT genotype group (P_{95}=73.3%), but are also different between the CC (P_{95}=2.8%), and the CT (P_{95}=9.1%) group. Thus, the CT genotype on average represents at most only a mild intermediate phenotype in terms of RBC nonmethylfolate accumulation, but occasional moderately elevated values for this ratio (5–15%) do occur in this group. In the MTHFR 677 CC and CT subjects, but not in the TT subjects, nonmethylfolate accumulation is positively correlated to total folate status, but with a relatively weak strength of correlation (r=.21, P=.04). Multivariate analysis of the combined CC and CT genotype group identifies the T-allele and total folate status as significant independent determinants of nonmethylfolate accumulation. The rather striking interindividual variability in RBC nonmethylfolate accumulation in subjects with the MTHFR 677 TT genotype is not explained by any of the variables we studied. In particular, vitamin B_2 status and *S*-adenosylmethionine, which are regulators of MTHFR activity on top of the 677 CT polymorphism [17,18], did not explain why some of these individuals have only minor amounts of RBC nonmethylfolate (i.e., approximately 10%), whereas in others, the RBC nonmethylfolate/total folate ratio can be as high as 70%.

The propensity to store RBC folate as nonmethylfolate in subjects with the MTHFR 677 TT genotype has been reported before. The first report, using HPLC coupled to electrochemical detection of intact polyglutamate isoforms of RBC folate, found on average 29% of RBC folate to consist of nonmethylfolate [7], which is remarkably similar to our finding of a median of 30.3%. However, these authors found no evidence of (even minor) RBC nonmethylfolate accumulation in MTHFR 677 CC subjects, nor in a preliminary analysis of a small number of CT genotype individuals. A subsequent study by the same group in a larger number of MTHFR 677 CC and TT genotype subjects confirmed their previous findings [9]. More recently, RBC folate isoforms were studied by HPLC–tandem mass spectrometry in 38 subjects (MTHFR C677T genotype distribution: 24 CC, 8 CT and 6 TT). Again, substantial but highly variable fractions of nonmethylfolate were found in the TT genotype subjects. In addition, a small (10%) but relatively consistent fraction of RBC folate in the MTHFR 677 CC and CT genotype subjects in this study consisted of nonmethylfolate, with no apparent difference between the CC and the CT genotype, although numbers in the latter subgroup were small (n=8) [6].

Compared with these previous reports, our study confirms that a considerable but highly variable degree of nonmethylfolate accumulation occurs in RBCs of MTHFR 677 TT genotype subjects. In addition, we have now characterized the RBC folate phenotype in a larger number of MTHFR 677 CT subjects and found that the CT genotype represents on average at most a very mild intermediate phenotype with respect to nonmethylfolate accumulation, the physiological ramifications of which are uncertain. It is not generally thought that the CT genotype is associated with alterations in cellular folate handling compared with the CC genotype. Insofar as our results suggest that this might still be the case, the explanation could be that the CT genotype is in fact associated with a moderate decrease in MTHFR enzyme activity (~71% residual enzyme activity relative to the CC genotype), albeit not as pronounced as in the TT genotype (~34% residual activity) [19].

In addition, we have studied other potential determinants of the interindividual variability in the RBC nonmethylfolate/total folate ratio in the different MTHFR C677T genotype strata. Of the variables studied, none appeared to affect nonmethylfolate accumulation, except total folate status. One interpretation of this result is that reduction of methylenetetrahydrofolate to 5-MTHFR is the central rate-limiting step in this part of the folate cycle. This is plausible because, in terms of diversion of metabolism to nonmethylfolates, this reaction is apparently sensitive to even mild reductions in activity of the MTHFR enzyme, such as in the 677 CT genotype [19]. In accordance with previous studies, a similar impact of total folate status on RBC nonmethylfolate accumulation is not apparent in the MTHFR 677 TT subjects [8,9]. It is possible that the effect of total folate status in this subgroup is simply overruled by the more pronounced reduction in MTHFR enzyme activity associated with the TT genotype. Alternatively, this finding may be due to a lack of statistical power because only 15 MTHFR 677 TT subjects participated in this study.

If our findings of progressive cellular nonmethylfolate accumulation with increased folate status are indeed correct, then this may have implications for how we are to understand the biological effects of folate supplementation. In patients with hyperhomocysteinemia, folic acid (pteroylmonoglutamic acid) is given, often in high doses, with the purpose of reducing homocysteine levels. However, for folate to regenerate methionine from homocysteine, it must be converted to 5MTHF first. The more folate is converted to nonmethylfolates, the less the relative effect on the methylation cycle is likely to be. In addition, nurturing of the DNA biosynthesis cycle by formation of formylated tetrahydrofolates in subjects using high-dose folate supple-

ments (usually in the form of folic acid) may underlie some of the — both positive and negative — effects of folate on other than homocysteine-related disorders. In particular, the effects on carcinogenesis (or, rather, tumor growth rate) [20] may be dependent on formation of formylated folates. To assess whether the effect of total folate status on nonmethylfolate accumulation, particularly in the MTHFR 677 CC and CT genotype subjects, can be extrapolated to persons with a very high folate status, the important next step would be to assess nonmethylfolate accumulation in people using high-dose folate supplements.

Other components of one-carbon metabolism were studied with respect to their potential effect on RBC nonmethylfolate accumulation, but none were found to be relevant in this respect. In particular, both plasma and RBC levels of *S*-adenosylmethionine, a known regulator of MTHFR activity [17,21], did not influence the RBC nonmethylfolate/total folate ratio. In addition, vitamin B_2 status, also important in the regulation of MTHFR activity [22], had no effect. Some studies have indicated that vitamin B_2 status is particularly important in individuals with the MTHFR 677 TT genotype [18,23] and/or with low folate status [23], and the mechanism behind this interaction has recently been elucidated [24]. In our study, there may well have been insufficient representation of subjects with the MTHFR TT genotype and sufficiently low folate status to exclude an effect of riboflavin on folate distribution.

An important advantage of our study is that we have used a relatively elaborate but highly sensitive and precise analytical method for measuring the folate vitamers. This allowed for identification of the mild but significant elevations in the RBC nonmethylfolate/total folate ratio in MTHFR 677 CT compared with CC subjects. Also, we performed a relatively extensive biochemical analysis to study potential determinants of folate vitamer distribution other than the MTHFR C677T genotype.

Limitations of our study include the fact that folate fractions were measured in RBCs, which are inactive in terms of homocysteine remethylation and DNA biosynthesis. Probably, RBC folate vitamer distribution is a reflection of what has occurred in RBC precursor cells in bone marrow. However, RBC folate vitamer distribution need not be representative of folate distribution in other cell types, particularly those with functioning remethylation and DNA/RNA biosynthesis pathways. Theoretically, measurement of folate distribution in other human cell types obtained in vivo should be possible with our technique, which requires small sample volumes, and we are currently exploring this possibility. Finally, our study is relatively limited in its statistical power to identify less dominant determinants of nonmethylfolate accumulation than the MTHFR C677T genotype. Neither subtle effects of other B vitamins, for example, nor concurrent effects of other common gene polymorphisms for other enzymes involved in folate metabolism be definitively excluded.

In conclusion, the MTHFR TT genotype is the most important determinant of nonmethylfolate accumulation in RBCs. In addition, high total folate status may cause moderate degrees of RBC nonmethylfolate accumulation in MTHFR CC and CT subjects.

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