

ORIGINAL ARTICLE

Red cell N^5 -methyltetrahydrofolate concentrations and C677T methylenetetrahydrofolate reductase genotype in patients with stroke

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J Clin Pathol 2004;57:54–57

Aims: To investigate the relation between total red cell folate, red cell N^5 -methyltetrahydrofolate (N^5 MTHF) concentrations, and N^5N^{10} -methylenetetrahydrofolate reductase (MTHFR) genotypes in stroke.

Methods: The study comprised 120 consecutive patients presenting to hospital with acute stroke. Multivitamin supplement use was recorded. Serum and red cell folate were measured by microbiological assays using *Lactobacillus casei* and *Enterococcus faecalis*, and by the DPC-BioMediq Immulite™ 2000 analyser. Total plasma homocysteine (tHcy), serum cobalamin, and serum vitamin B₆ were measured and the C677T MTHFR genotype determined.

Results: There were no significant differences in blood tHcy or vitamin concentrations according to MTHFR genotype in the overall patient cohort. However, when patients taking vitamins were excluded, total red cell folate and red cell N^5 MTHF were significantly lower in patients with the TT genotype compared with CT or CC genotypes. In the overall cohort, irrespective of genotype, red cell folate was significantly lower when assayed microbiologically than with the Immulite assay. This discrepancy remained after exclusion of patients taking vitamins.

Conclusion: Total red cell folate and red cell N^5 MTHF are significantly lower in stroke patients with the TT compared with the CT and TT MTHFR genotypes, particularly those not taking vitamin supplements. Microbiological assays that measure biologically active folates provide substantially lower estimates of folate than the Immulite™ assay. Because folate is a key determinant of blood homocysteine values, these findings may impact on the interpretation of the strength and independence of the association between raised blood concentrations of homocysteine and atherothrombosis risk reported in most epidemiological studies.

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Accepted for publication
28 July 2003

Moderately raised blood concentrations of total homocysteine (tHcy) are an independent risk factor for atherothrombotic vascular disease, including stroke.^{1,2} However, it remains uncertain whether high homocysteine causes atherothrombosis or whether the disease may be caused by a different component of the homocysteine metabolic cycle.

In most tissues, homocysteine is re-methylated back to methionine by methionine synthase, with N^5 -methyltetrahydrofolate reductase (MTHFR) acting on N^5 -methyltetrahydrofolate (N^5 -MTHF), which becomes a methyl group donor, and cobalamin an essential cofactor.³ The formation of N^5 MTHF depends on the presence of N^5N^{10} -methylenetetrahydrofolate and the enzyme MTHFR. Absent or reduced function of any of these components might be a cause of atherothrombosis.

“In association with poor folate status, MTHFR mutation has been linked with raised total homocysteine and an increased risk of atherothrombotic vascular disease”

A substantial proportion of the population has a common point mutation (C to T substitution at nucleotide 677 (C677T)) in the coding region of the gene for MTHFR, which is associated with a thermolabile variant that has reduced activity (by about 50%). In association with poor folate status, this mutation has been linked with raised tHcy and an increased risk of atherothrombotic vascular disease.^{4–7} The relation between tissue folate stores and tHcy for the three

different MTHFR genotypes—CC, CT, and TT—has also been investigated.^{8–12} However, to date, results have been inconsistent, with either raised or reduced red cell folate reported in association with the homozygous TT genotype. These inconsistent results might be explained, at least in part, by variations in laboratory methods used and the demographics of the populations under investigation. Molloy *et al* compared red cell folate values obtained by radioassay and microbiological assay.¹³ In two different populations they found that the red cell folate was consistently higher in the CC and CT groups when measured by the microbiological assay compared with the radioassay, whereas in the TT groups, the microbiological assay revealed lower red cell folate values. Quere *et al*, using a microbiological assay, reported a strong association between red cell N^5 MTHF, but not plasma folate, and the C677T mutation in patients with deep vein thrombosis.¹⁴

The aim of our study was to investigate the relation between total red cell folate, red cell N^5 MTHF values, and the MTHFR genotypes found in patients with stroke. We hypothesised that patients with stroke who have the TT genotype would have lower red cell N^5 MTHF than those with the CC and CT genotypes, particularly patients not taking folate supplementation.

Abbreviations: N^5 MTHF, N^5 -methyltetrahydrofolate; MTHFR, N^5N^{10} -methylenetetrahydrofolate reductase; tHcy, total homocysteine

Table 1 Homocysteine and vitamin status in all patients with stroke according to MTHFR genotype

Variable	Genotype (three categories)			p Value (ANOVA)	Genotype (two categories)		p Value (ANOVA)
	CC (N=55)	CT (N=52)	TT (N=13)		CC/CT (N=107)	TT (N=13)	
Plasma homocysteine ($\mu\text{mol/l}$)	11.2	11.2	11.9	0.80	11.2	11.9	0.51
Serum B ₆ (nmol/l)	38.1	46.5	46.6	0.22	41.7	46.1	0.59
Serum cobalamin (pmol/l)	468.7	411.6	437.0	0.31	441.4	437.0	0.97
Total red cell folate (nmol/l)	652.0	692.3	523.2	0.28	671.8	523.2	0.13
Red cell N ⁵ MTHF (nmol/l)	626.4	665.1	497.7	0.26	645.5	497.7	0.12
Serum folate (nmol/l)	18.0	22.2	17.8	0.46	19.9	17.8	0.67
Serum N ⁵ MTHF (nmol/l)	17.3	20.9	15.2	0.38	18.9	15.2	0.41
"Immunoassay" red cell folate (nmol/l)	916.0	962.9	1064.2	0.69	943.9	1064.2	0.45

N⁵MTHF, N⁵-methyltetrahydrofolate; MTHFR, N⁵N¹⁰-methylene tetrahydrofolate reductase.

MATERIALS AND METHODS

Patients

Our study comprised 120 consecutive patients presenting to hospital with acute stroke between September 2001 and June 2002. Our study was approved by the local institutional ethics committee and all patients provided written consent. The use of vitamin supplements at the time of hospitalisation was recorded for each patient.

Sample collection and laboratory assays

Venous blood samples were collected after an overnight fast for serum and red cell total folate assay, serum and red cell N⁵MTHF assay, serum cobalamin assay, tHcy concentration, and MTHFR genotyping. tHcy was determined on the Abbott IMxTM analyser. Folate was measured by three separate methods, namely: (1) an automated microbiological assay using a chloramphenicol resistant strain of *Lactobacillus casei* as the test organism¹⁵; (2) the DPC BioMediq ImmuliteTM 2000 analyser using a chemiluminescent enzyme immunoassay; and (3) a microbiological assay with *Enterococcus faecalis*. *Enterococcus faecalis* responds to a similar range of folate species to *L casei*, with the notable exception of N⁵MTHF. N⁵MTHF was determined by subtracting the result obtained with *E faecalis* from the *L casei* result.¹⁶ The serum cobalamin concentration was determined by microbiological assay using *Euglena gracilis* as the test organism.¹⁷ Serum vitamin B₆ was determined by microbiological assay using *L casei* as the test organism.¹⁸ Vitamin B₆ was measured to eliminate vitamin B₆ deficiency as a cause of raised serum homocysteine. Serum creatinine was measured on a Hitachi 917 analyser.

Red cell samples for total folate and N⁵MTHF assays were prepared essentially using the method of Wright *et al*,¹⁹ which involves the use of a lysing agent during preparation of the haemolysate. The prepared haemolysates were stored at

-20°C and assayed in batches once sufficient samples had been collected. On the day of the assay, haemolysates were thawed at 37°C, well mixed, and centrifuged at 1500 ×g for 10 minutes to remove any cell stroma. The supernatant was then recovered and assayed in duplicate.

Genotyping for the MTHFR gene was performed using polymerase chain reaction followed by restriction fragment length polymorphism on DNA that spans nucleotide 677 of the MTHFR gene.²⁰ This was done independently of the vitamin assays and the patients' genotypes were not revealed until all the other assays had been completed.

Statistical analysis

Logarithmic transformations were used for variables showing a positive skew and are presented as geometric means. Blood concentrations of homocysteine and vitamins were compared in the three patient groups according to MTHFR genotype using analysis of variance (ANOVA), both adjusted and unadjusted for age, sex, and serum creatinine. These analyses were performed separately in the overall patient cohort and after excluding patients taking vitamin supplements at hospital admission or at the time of the blood collections.

RESULTS

One hundred and twenty patients with stroke were studied, 75 (62.5%) of whom were taking vitamin supplements at the time of blood sampling.

The distribution of the C677T genotypes in our patients was similar to that reported in previous studies of patients with vascular disease,^{4 21 22} with CC comprising 46%, CT 43%, and TT 11% of the cohort. A similar distribution has also been reported in the general population.¹³

Table 1 shows the vitamin and homocysteine concentrations for all 120 patients according to C677T genotype and table 2 shows the same results but excluding patients taking

Table 2 Homocysteine and vitamin status in patients with stroke who have different MTHFR genotypes after excluding patients taking vitamin supplements

Variable	Genotype (three categories)			p Value (ANOVA)	Genotype (two categories)		p Value (ANOVA)
	CC (N=21)	CT (N=24)	TT (N=4)		CC/CT (N=45)	TT (N=4)	
Plasma homocysteine ($\mu\text{mol/l}$)	13.9	11.5	12.6	0.17	12.4	12.6	0.96
Serum B ₆ (nmol/l)	31.5	37.3	32.1	0.50	34.8	32.1	0.77
Serum cobalamin (pmol/l)	419.9	376.2	528.5	0.21	395.4	528.5	0.14
Total red cell folate (nmol/l)	645.5	589.9	273.1	0.02	614.0	273.1	0.01
Red cell N ⁵ MTHF (nmol/l)	614.0	566.8	252.1	0.02	589.9	252.1	<0.01
Serum folate (nmol/l)	12.2	18.2	9.2	0.12	15.0	9.2	0.23
Serum N ⁵ MTHF (nmol/l)	12.1	17.3	9.1	0.16	14.6	9.1	0.26
"Immunoassay" red cell folate (nmol/l)	727.8	888.9	658.5	0.32	812.4	658.5	0.43

N⁵MTHF, N⁵-methyltetrahydrofolate; MTHFR, N⁵N¹⁰-methylene tetrahydrofolate reductase.

vitamin supplements at the time of admission to hospital or at the time of blood collection.

There were no significant differences in blood concentrations of tHcy or vitamins according to MTHFR genotype in the overall patient cohort. However, when patients taking multivitamins were excluded, total red cell folate and red cell N^5 MTHF were significantly lower in patients with the TT genotype compared with the other two groups. Results were not altered after adjustment for age, sex, and creatinine; therefore, only the unadjusted results are presented.

There was a substantial discrepancy between blood concentrations of total folate in the overall cohort of patients, irrespective of genotype, when measured by the microbiological assays and the Immulite™ analyser. This discrepancy remained evident after exclusion of patients taking vitamin supplements.

DISCUSSION

Our results show that patients with the TT MTHFR genotype have substantially lower total red cell folate and N^5 MTHF concentrations than those with the CT and CC genotype. This difference was masked by the use of multivitamins. There was also a significant discrepancy between total red cell folate when measured by microbiological assays and the Immulite™ analyser, with the last method yielding mean concentrations of red cell folate that were approximately twice as high as those measured by the microbiological assays in patients with the TT MTHFR genotype.

Molloy *et al* previously reported that microbiological assays provide significantly lower estimates of red cell folate concentrations in patients with the TT genotype than do radioassay techniques.¹³ This is similar to the results of our study comparing microbiological assays with the Immulite™ method. Surprisingly, however, Molloy and colleagues¹³ reported higher red cell folate concentrations in the CT and CC genotypes when assayed by microbiological assays than with the radioassay technique, which is in contrast to our findings.

“Our data demonstrate the potential masking effects of multivitamin supplementation on the association between the common C677T genotypes and red cell folate concentration”

Microbiological assays measure biologically active folate species and one explanation for the discrepancy with other assay techniques could be that the immunological assay is responding to a species of folate that is not biologically active in the cases concerned. However, this cannot explain the apparently conflicting results between the results of Molloy *et al* and our study in patients with the CT and CC genotypes. More detailed investigation of the range of folate species present is required to elucidate these issues.

Despite a large body of epidemiological evidence indicating an independent association between moderately raised blood concentrations of tHcy and atherothrombosis risk,³ some studies have also suggested an association between low folate concentrations or intake and atherothrombotic vascular disease that is independent of homocysteine.²³ Our data are important in this context, because they not only demonstrate the potential masking effects of multivitamin supplementation on the association between the common C677T genotypes and red cell folate concentration, but also suggest that microbiological assays that measure biologically active species of folate may provide a more sensitive estimate of the association between folate status and atherothrombosis risk. This hypothesis should be investigated in laboratory and clinical studies that examine the association between

Take home messages

- Total red cell folate and red cell N^5 -methyltetrahydrofolate are significantly lower in patients with stroke who have the TT MTHFR (N^5N^{10} -methyltetrahydrofolate reductase) genotype than in those with the CT and TT genotypes, particularly in those not taking vitamin supplements, which can mask this association
- Microbiological assays that measure biologically active folates provide substantially lower estimates of folate than the Immulite™ assay, and may provide a more sensitive estimate of the association between folate status and atherothrombosis risk
- Folate is a key determinant of blood homocysteine values, so that these findings may impact on the interpretation of the strength and independence of the association between raised blood concentrations of homocysteine and atherothrombosis risk reported in most epidemiological studies

biologically active folates and atherothrombosis risk, and a more extensive study, including sex and aged matched non-stroke patients, is warranted.

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ECHO

Patients have no appetite for HSV-2 screening



Please visit the *Journal of Clinical Pathology* website [www.jclinpath.com] for a link to the full text of this article.

Halting the spread of herpes simplex virus type 2 (HSV-2) in the UK remains a distant prospect, after a pilot study has shown that uptake of a type specific herpes antibody test would be too low to be effective.

The study examined attitude to theoretical and actual testing and uptake of the test among men and women attending a genitourinary (GUM) clinic in a district general hospital in southeast England.

About half of the patients—whether new consecutive attendees (207) or reattendees (205)—said they would have liked the test if had been available. In another group of 434 consecutive attendees only about 40% of men or women wished to take up the offer of actual testing. Refusal was explained variously by dislike of blood tests; no time for counselling, getting the results, or both; ambivalence about the result; and known acute genital herpes infection.

In the event, 38% of patients had the test, and 16 new cases of HSV-2 were diagnosed—way below the 72 new cases calculated from the known ratio of HSV-1 to HSV-2 infection in this population and the proportion likely to be diagnosed.

HSV-2 infection is more commonly seen in GUM clinics than among blood donors, women, and homosexual men. Infected persons tend to be older and unaware of the infection, increasing the likelihood of spread, given the propensity of HSV-2 for asymptomatic shedding. A preliminary study in Leeds in the late 1990s suggested strong support among patients for HSV-2 screening.

▲ *Sexually Transmitted Infections* 2003;**79**:129–133.



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J Clin Pathol 2004 57: 54-57
doi: 10.1136/jcp.57.1.54

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