

# Effect of low doses of 5-methyltetrahydrofolate and folic acid on plasma homocysteine in healthy subjects with or without the 677C→T polymorphism of methylenetetrahydrofolate reductase

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## Abstract

**Background** The 677C→T polymorphism of methylenetetrahydrofolate reductase can lead to increased homocysteine. Moderate increases of homocysteine can be lowered by folic acid (0.4–10 mg day<sup>-1</sup>). This study compared the effect of folic acid with 5-methyltetrahydrofolate, the active form of folate generated by this reductase, on homocysteine levels in healthy subjects and whether this is influenced by the 677C→T polymorphism.

**Materials and methods** Either 400 µg day<sup>-1</sup> of [6RS] 5-methyltetrahydrofolate or 400 µg day<sup>-1</sup> of folic acid were administered orally to 10 wild-type and 10 homozygous subjects. Total homocysteine and folate were determined before and after 3 and 7 weeks of treatment, and 24 weeks after stopping treatment.

**Results** After 3 and 7 weeks of treatment with 5-methyltetrahydrofolate, homocysteine levels fell from 11.6 ± 1.5 to 9.0 ± 2.3 and 8.7 ± 1.8 ( $P < 0.005$ ) in wild-type subjects and from 16.9 ± 6.8 to 12.3 ± 4.3 and 11.6 ± 4.4 µmol/L, mean ± SD ( $P < 0.005$ ) in homozygous subjects, proving biological availability of 5-methyltetrahydrofolate irrespective of the 677C→T genotype.

After folic acid for 3 and 7 weeks, values fell from 12.6 ± 3.3 to 9.2 ± 2.9 and 9.2 ± 2.7 ( $P < 0.005$ ) and from 15.6 ± 4.9 to 11.7 ± 3.9 and 9.1 ± 2.4 µmol L<sup>-1</sup>, mean ± SD ( $P < 0.005$ ) in wild-type and homozygous subjects, respectively.

Six months after stopping treatment, homocysteine levels remained lower than pre-treatment levels, with statistical significance, only in homozygous subjects treated with 5-methyltetrahydrofolate (12.1 ± 2.5 vs. 16.9 ± 6.8,  $P < 0.01$ ).

**Conclusions** 5-methyltetrahydrofolate showed comparable efficacy in reducing homocysteine as folic acid. A prolonged effect 6 months after ceasing treatment with 5-methyltetrahydrofolate in homozygous subjects represents a further phenotypic effect of the 677TT methylenetetrahydrofolate reductase genotype.

**Keywords** 5-methyltetrahydrofolate, folic acid, homocysteine, methylenetetrahydrofolate reductase, polymorphism.

*Eur J Clin Invest* 2002; 32 (9): 662–668

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Received 25 February 2002; accepted 6 June 2002

## Introduction

A large number of retrospective and prospective studies have shown that moderately elevated plasma homocysteine is associated with increased risk for vascular disease [1–4]. Homocysteine is now well established as an independent risk factor for various types of vascular disease, including coronary arterial, peripheral arterial and cerebrovascular occlusion, as well as thrombotic disease, although the exact causal relationship remains to be proven [5,6].

Vitamin B12-dependent remethylation of homocysteine to methionine is catalysed by methionine synthase which utilises 5-methyltetrahydrofolate (5-MeTHF) as the primary methyl donor. 5-MeTHF is formed from 5,10-methylene-tetrahydrofolate by 5,10-methylenetetrahydrofolate reductase (MTHFR) [7]. In the trans-sulfuration pathway, homocysteine is converted to cystathionine by cystathionine  $\beta$ -synthase. Inherited defects of these three key enzymes lead to severely disturbed homocysteine metabolism.

Nutritional factors influence homocysteine levels: for example, plasma concentrations of folate, vitamin B<sub>12</sub> and B<sub>6</sub> are inversely correlated with plasma total homocysteine [8,9].

The thermolabile variant of MTHFR [10], caused by a C to T substitution at nucleotide 677 which converts an alanine to a valine residue [11], also leads to higher homocysteine levels, particularly when the folate status is inadequate. Thus individuals who are homozygous for the 677C→T polymorphism exhibit lower activity and reduced stability of MTHFR at 46 °C [11]. The resulting reduced production of 5-MeTHF leads to changes in cellular composition of one-carbon folate derivatives [12].

Folic acid has been shown to lower homocysteine in many studies [13]: doses ranging from 0.4 mg to 10 mg day<sup>-1</sup>, sometimes combined with vitamins B6 and/or B12, lowered total homocysteine levels by 16–39% of initial levels.

The efficacy of folic acid in lowering homocysteine might be influenced by the MTHFR 677C→T genotype [14].

Administration of 5-MeTHF should bypass the several steps involved in conversion of folic acid to its active co-enzyme form (Fig. 1), and might be more efficient in lowering homocysteine.

Only a few studies have reported on the treatment of hyperhomocysteinaemia with 5-MeTHF. Oral administration of supra-physiological doses (15 mg day<sup>-1</sup>) for two months markedly reduced homocysteine levels in end-stage renal disease patients [15], while the homocysteine lowering

effect of oral 5-MeTHF (17 mg day<sup>-1</sup>) was shown to be equal to that of folic acid (15 mg day<sup>-1</sup>) in patients on hemodialysis [16]. Doshi *et al.* [17] showed that intra-arterial infusion of 5-MeTHF, for just 30 min, improved endothelial function measured as flow-mediated dilation without any concomitant fall of homocysteine. *In vitro*, 5-MeTHF was more effective than folic acid in reducing homocysteine export in human umbilical vein endothelial cells [18].

One difficulty in assessing the efficacy of 5-MeTHF vs. folic acid is the availability of 5-MeTHF as a mixture of the [6R] and [6S] racemates. It is assumed that only the [6S] form, which is half the administered dose, is bio-active. However evidence exists that the [6R] racemate of 5-formyltetrahydrofolate can also be biologically active, although it is not known whether this also applies to the [6R] form of 5-MeTHF [19–21].

This study set out to test the effect of nutritional doses of 5-MeTHF compared with folic acid on homocysteine levels in healthy subjects, with and without the 677C→T polymorphism.

## Methods

Forty healthy Caucasian subjects, 32 males and eight females, between 19 and 69 years of age with a fasting plasma total homocysteine level above 10  $\mu\text{mol L}^{-1}$  but below 30  $\mu\text{mol L}^{-1}$  were recruited from Basel and its surrounding area, the majority from a blood donation centre. Of these, 20 were wild type and 20 homozygous for the 677C→T polymorphism.

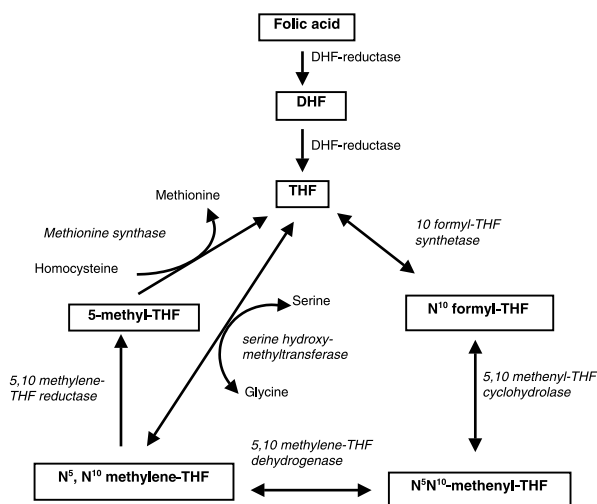
Inclusion criteria included body weight within  $\pm 20\%$  of normal values, no intake of B group or multivitamin preparations for at least 2 months before the study and normal health parameters including blood pressure, heart rate, haemoglobin, haematocrit, cholesterol, triglycerides, glucose, albumin, total protein, creatinine and alanine amino transferase.

At entry a complete medical history was taken and physical examination performed.

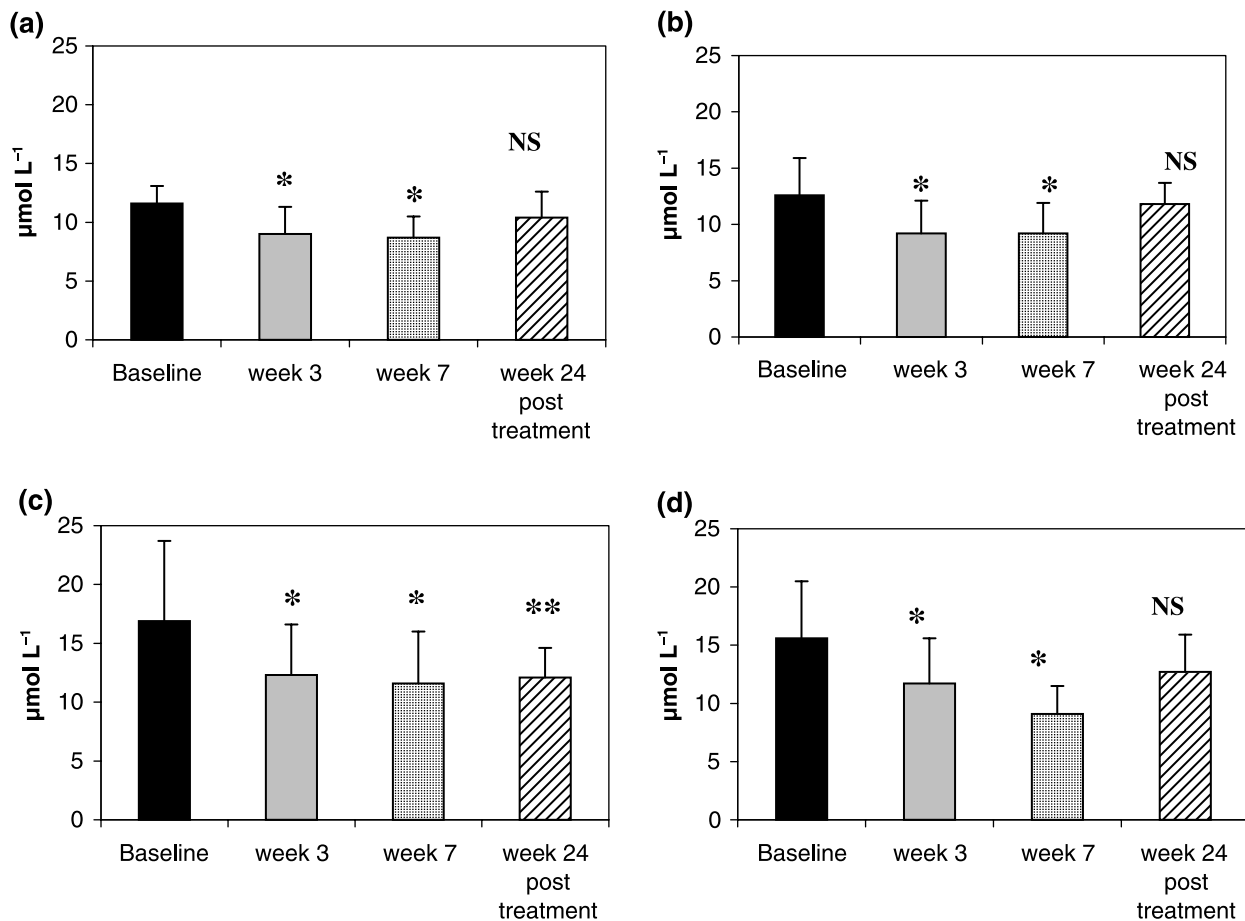
Subjects within the wild-type (677CC) and homozygous (677TT) groups were randomly selected for oral treatment with either 5-MeTHF or folic acid in a double-blind design. Ten subjects from each genotype group received 400  $\mu\text{g}$  daily of 5-MeTHF Ca salt, and the other 10 subjects were given folic acid for 7 weeks. 5-MeTHF was given as the [6RS] racemate form, which is equivalent to 200  $\mu\text{g}$  of the natural [S] isomer, supplied by Knoll Pharma, Italy. Folic acid (Nature's Bounty Inc.) was also supplied by Knoll Pharma, Italy.

Blood was collected fasting for determination of total plasma homocysteine and total serum folate at the start of the study, after 3 and 7 weeks of treatment and again 24 weeks after stopping treatment.

For homocysteine, blood was collected in EDTA tubes, separated within 30 min (2000 g 3400 r.p.m.<sup>-1</sup> 10 min<sup>-1</sup>, 4 °C) and plasma stored at -20 °C until analysis.



**Figure 1** The main reactions involved in the conversion of folic acid to 5-MeTHF in man are summarised. DHF, dihydrofolic acid; THF, tetrahydrofolic acid.



**Figure 2** Homocysteine levels immediately before, after 3 and 7 weeks of treatment and 24 weeks after ceasing treatment in the two genotype groups. (a) The wild-type group of subjects treated with 5-MeTHF; (b) the wild-type group of subjects treated with folic acid; (c) the homozygous group of subjects treated with 5-

MeTHF; and (d) the homozygous group of subjects treated with folic acid. Data are presented as mean + SD. Statistical significance of changes in homocysteine levels at each time point compared with the baseline value is indicated for each group as follows: \* $P = 0.005$ ; \*\* $P = 0.01$ ; NS = no statistical significance.

Total homocysteine was determined by high-pressure liquid chromatography (HPLC) after reduction followed by derivatization with ammonium 7-fluoro-benzo-2-oxa-1,3-diazole-4-sulphonate and fluorimetric detection as previously described [22].

Total folate was measured in serum treated by adding ascorbic acid ( $5 \text{ mg mL}^{-1}$ ) using the 'Access Folate' kit manufactured by Sanofi Diagnostics Pasteur, France.

5-MeTHF was determined in plasma by HPLC with fluorescence detection as previously described [23].

MTHFR 677C→T genotyping was performed on DNA isolated from EDTA blood with the QIAamp kit (Qiagen, Basel, Switzerland) using a previously described polymerase chain reaction-based procedure [11].

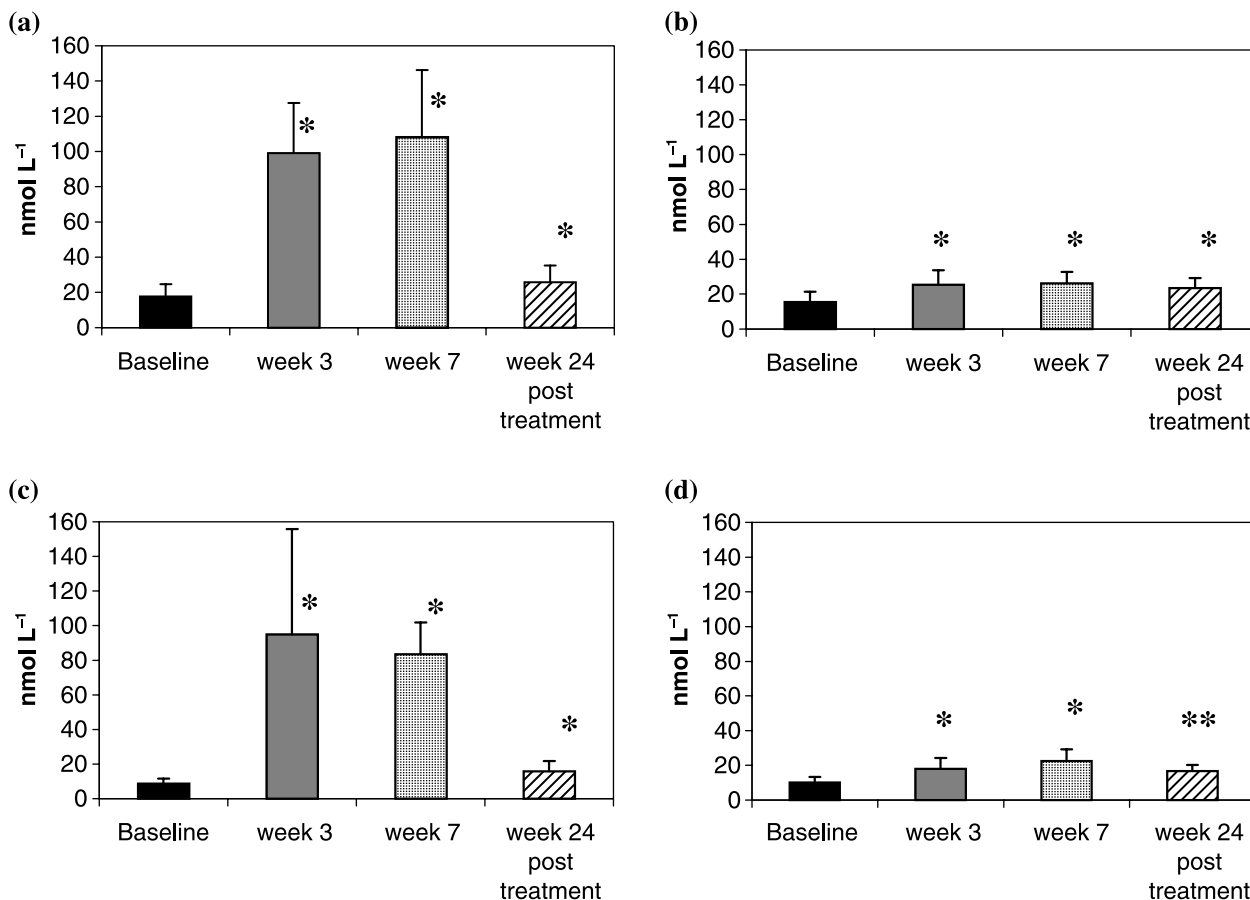
Statistical analysis was performed as follows. Total plasma homocysteine before and after treatment within the four groups was analysed by the Wilcoxon signed-rank paired test. Statistical significance of differences between the groups was calculated by the Mann-Whitney U-test.

The study was approved by the Basel University Ethical Committee and eligible subjects gave written informed consent for participation.

## Results

In comparing the characteristics of 677CC and 677TT subjects, no statistical differences were observed for age, sex and health parameters between the two genotype groups except that body weight was slightly higher in the 677CC subjects ( $83 \pm 9.1$ ) than in the 677TT subjects ( $78.5 \pm 8.9$ ,  $P = 0.01$ ). The predominance of males in both groups reflects the pattern within our recruitment population.

Before treatment total homocysteine levels were higher in the 677TT group ( $n = 20$ ,  $16.2 \pm 5.8 \text{ } \mu\text{mol L}^{-1}$ ) than in the 677CC group ( $n = 20$ ,  $12.1 \pm 2.5 \text{ } \mu\text{mol L}^{-1}$ ), with statistical significance ( $P = 0.003$ ). Also folic acid levels were lower in the 677TT group ( $n = 20$ ,  $9.5 \pm 3.1 \text{ nmol L}^{-1}$ ) than in the



**Figure 3** Folate levels immediately before, after 3 and 7 weeks of treatment and 24 weeks after ceasing treatment in the two genotype groups. (a) The wild-type group of subjects treated with 5-MeTHF; (b) the wild-type group of subjects treated with folic acid; (c) the homozygous group of subjects treated with 5-MeTHF; and

(d) the homozygous group of subjects treated with folic acid. Data are presented as mean + SD. Folate was measured as 5-MeTHF at 24 weeks post treatment. Statistical significance of changes in folate levels at each time point compared with the baseline value is indicated for each group as follows: \* $P = 0.005$ ; \*\* $P = 0.007$ .

677CC group ( $n = 20$ ,  $16.6 \pm 6.2$  nmol L<sup>-1</sup>), with statistical significance ( $P < 0.0001$ ).

Total homocysteine and folate values in the two genotype groups at baseline and at different times following treatment with either 5-MeTHF or folic acid are illustrated in Figs 2 and 3.

In each of the four groups there was a clear statistically significant fall of total homocysteine, reaching 73–78% of initial values after treatment for 3 weeks. Total homocysteine levels remained virtually the same after a further 4 weeks' administration of either treatment in the 677CC subject groups. However levels fell further in the 677TT groups, reaching 58% and 69% of initial values after treatment with folic acid and 5-MeTHF, respectively.

Twenty-four weeks after ceasing treatment homocysteine levels returned towards baseline values in each group apart from the 677TT subjects treated with 5-MeTHF.

Thus although levels did not return completely to those before treatment, there was no statistical difference between the values 24 weeks after stopping treatment and those at baseline in the wild-type subjects on both treatments and

the homozygous subjects on folic acid. In contrast the mean level of total homocysteine remained at 71% of the initial value ( $P < 0.01$ ) in the 677TT subjects treated with 5-MeTHF.

When comparing the two treatments there were no statistically significant differences of homocysteine values between the groups receiving folic acid or 5-MeTHF at any time post treatment in both the wild-type and homozygous groups. When comparing the genotype groups, after folic acid there were no statistical differences between homocysteine values in the 677CC and 677TT subjects. After 5-MeTHF, homocysteine levels were somewhat higher in the 677TT group, reflecting the higher baseline values (pre-treatment,  $P = 0.02$ ; week 3,  $P = 0.003$ ; week 7,  $P = 0.05$ ; 24 weeks' post treatment, NS).

The effect of treatment on folate levels is shown in Fig. 3. Moderate increases of folate levels were observed in both genotype groups after administration of folic acid. Thus levels at 7 weeks were 68% and 120% higher than initial values after folic acid in the 677CC group and 677TT group, respectively. In contrast approximately six- and

10-fold increases of folate were achieved in the 677CC group and 677TT group, respectively, after treatment with 5-MeTHF. After ceasing treatment for 24 weeks the high levels seen in the 5-MeTHF-treated subjects fell considerably, almost reaching baseline levels. Folate levels were highly significantly different after 5-MeTHF compared with folic acid at 3 weeks and 7 weeks ( $P = 0.0002$ ) but not different at 24 weeks post treatment in both genotype groups.

Comparing the two genotype groups, there were no statistical differences between folate levels in the 677CC compared with the 677TT subjects after 3 and 7 weeks of both treatments. As seen before treatment, folate levels at 24 weeks post treatment were higher in the 677CC than in the 677TT groups (folic acid treatment,  $P = 0.02$ ; 5-MeTHF treatment  $P = 0.005$ ).

## Discussion

This double-blind study revealed two main new findings. First, the ability of a physiological dose of 5-MeTHF to reduce plasma total homocysteine to a similar degree to that obtained with daily administration of 400 µg folic acid in healthy adult subjects. Second, there was evidence of a prolonged effect 6 months after ceasing treatment in subjects with the 677TT MTHFR genotype, with clear statistical significance, in those treated with 5-MeTHF.

An important feature of this study was the comparison of subjects classified according to the 677C→T genotype of MTHFR. Although the total group was selected according to clearly defined minimum and maximum total homocysteine levels, the inherent impact of the MTHFR polymorphism was evident in that higher total homocysteine and lower folate levels were found in the 677TT subjects before any treatment. In this context Malinow *et al.* reported differences in response of plasma homocysteine levels to 1–2 mg day<sup>-1</sup> of folic acid supplementation between homozygous and wild-type individuals [24].

The degree of reduction of homocysteine that we found after either folic acid or 5-MeTHF is similar to that observed in previous studies. Thus the reduction of 27% we found in wild-type subjects treated with 0.4 mg folic acid day<sup>-1</sup> is similar to the reduction of 25% in subjects with initial homocysteine values of 11.0–13.6 µmol L<sup>-1</sup> reported in the meta-analysis of randomised trials of folic acid treatment [13]. Similarly, the reduction of 42% in the 677TT subjects on folic acid compares with a reduction of 28% with initial values in the range 13.7–18.5 in the meta-analysis. This greater degree of reduction of homocysteine with folic acid in 677TT subjects, seen only after 7 weeks, probably reflects higher initial homocysteine levels and possibly altered folate pools.

Previous studies have used folic acid doses ranging from 0.4 to 10 mg day<sup>-1</sup> with no obvious further effect of higher doses, suggesting that a maximum homocysteine lowering effect is possible with doses close to levels used in this study, which are similar to those advocated for fortification of food

as a means of lowering homocysteine concentrations in the general population [25].

The administration of 5-MeTHF led to a reduction of homocysteine to a similar degree to that obtained with folic acid. This finding confirms the bio-availability of this co-enzyme form, which participates directly in the remethylation of homocysteine catalysed by methionine synthase.

The degree of homocysteine reduction of 22–42% achieved in this study with a physiological dose of 5-MeTHF was higher than the 17% reduction found in patients with renal disease treated with the much higher pharmacological dose of 17 mg day<sup>-1</sup> [16]. Significantly greater reductions of homocysteine (average 72%) were reported using 15 mg day<sup>-1</sup> 5-MeTHF in uraemic haemodialysis patients [15], although initial homocysteine levels were much higher than those reported by Bostom *et al.* [16] and those in our subjects. As suggested by Bostom *et al.* [16] inherent differences in renal disease patients may make comparisons between healthy subjects and this disease population invalid.

The dose of 5-MeTHF that we used is important in assessing its efficacy compared with the dose of folic acid. We selected an equal amount of [6RS] 5-MeTHF to that of folic acid, recognising that only half of this, the [6S] isomer, is expected to be active as a substrate for folate enzymes. The natural form of folic acid was shown to be equally effective as double the amount of [6RS] 5-MeTHF in restoring growth in folate-depleted rats [26]. Conversely, absorption and transport may be equal for each isomer, as suggested by the observation that the reduced folate carrier of some murine tumour cell lines is not stereo-specific for 5-MeTHF [27].

In addition, 800 µg day<sup>-1</sup> of [6RS] 5-MeTHF containing 400 µg day<sup>-1</sup> of the active isomer may have exceeded the dynamic range of dose to response, as the active coenzyme could conceivably be more efficient than folic acid in reducing homocysteine.

It appears that the lowering effect of 200 µg day<sup>-1</sup> of [6S] 5-MeTHF is similar to that of 400 µg day<sup>-1</sup> of folic acid. However, the maximum effect may be possible with less than 400 µg day<sup>-1</sup>. In this regard Guttormsen *et al.* reported a lowering of elevated homocysteine to below 20 µmol L<sup>-1</sup> after treatment with 200 µg day<sup>-1</sup> folic acid in 21 of 37 subjects with intermediate hyperhomocysteinaemia [28].

An important finding in this study was the considerably higher folate levels after 5-MeTHF compared with levels achieved with folic acid.

The finding of folate levels after [6RS] 5-MeTHF at least as high as after folic acid suggests that both [R] and [S] 5-MeTHF are taken up. As folate levels were much higher after 5-MeTHF we speculate that the [R] form has a much lower rate of turnover than the [S] form. The exact processes resulting in this lower turnover are unclear.

The prolonged effect of 5-MeTHF in reducing homocysteine in 677TT subjects only, represents a further phenotypic effect of this genotype. We speculate that a shift in the disposition of reduced folates, towards 5,10 methylene-THF as a result of 677TT leads to a slower turnover of administered folate.

As [6R] 5-MeTHF is unlikely to be metabolised we speculate that it may inhibit regulatory enzymes related to homocysteine metabolism. For example, serine hydroxymethyltransferase [29], a critical enzyme in interconversion of folate coenzymes, is inhibited by 5-MeTHF. Whether this inhibition leads to a delayed turnover of folate coenzymes, which are influenced by the MTHFR 677C→T genotype, can only be speculated and requires further study.

Glycine N-methyl transferase, which is considered to play a major role in the removal of methyl groups and hence in the overall catabolism of methionine, has been shown to be inhibited by the [6S] isomer of 5-MeTHF-pentaglutamate [30]. However, inhibition by the [6RS] form was only approximately half that of an equal concentration of the [6S] form, indicating that the [6R] form of 5-MeTHF does not inhibit this methyl transferase.

Finally, the demonstration of bio-availability of 5-MeTHF indicates that this compound may be of benefit in the treatment of severe methylene-THF reductase deficiency.

## Acknowledgements

This work was supported by grants from the Swiss National Science Foundation (No. 3200-045988-98) and from Knoll Farmaceutici SpA (Milan, Italy) and Knoll-Bioresearch SA (S. Antonino, Switzerland).

We thank Drs C. Di Padova and P. Giulidori for their stimulating discussions and advice in preparing the manuscript.

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