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The common 'thermolabile' variant of methylene tetrahydrofolate reductase is a major determinant of mild hyperhomocysteinaemia

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Summary

Mild hyperhomocysteinaemia is a major risk factor for vascular disease and neural tube defects (NTDs), conferring an approximately three-fold relative risk for each condition. It has several possible causes: heterozygosity for rare loss of function mutations in the genes for 5,10-methylene tetrahydrofolate reductase (MTHFR) or cystathionine-β-synthase (CBS); dietary insufficiency of vitamin co-factors B6, B12 or folates; or homozygosity for a common 'thermolabile' mutation in the MTHFR gene which has also been associated with vascular disease and NTDs. We quantified the contribution of the thermolabile mutation to the hyperhomocysteinaemic phenotype in a working male population (625 individuals). Serum folate and vitamin B12 concentrations were also measured and their relationship with homocysteine status and MTHFR genotype assessed. The homozygous thermolabile genotype occurred in 48.4, 35.5, and 23.4% of the top 5, 10, and 20% of individuals (respectively) ranked by plasma homocysteine levels, compared with a frequency of 11.5% in the study population as a whole, establishing that the mutation is a major determinant of homocysteine levels at the upper end of the range. Serum folate concentrations also varied with genotype, being lowest in thermolabile homozygotes. The MTHFR thermolabile genotype should be considered when population studies are designed to determine the effective homocysteine-lowering dose of dietary folate supplements, and when prophylactic doses of folate are recommended for individuals.

Introduction

Mild hyperhomocysteinaemia is now recognized as an important risk factor for coronary artery disease (CAD),[1,2] peripheral vascular disease,[3,4] cerebrovascular disease[4–6] and recurrent venous thrombosis.[7] Elevated maternal levels of homocysteine have recently been associated with the occurrence of NTDs.[8]

Homocysteine is an unstable thiol amino acid generated solely as a product of transmethylation reactions which consume S-adenosyl methionine (SAM). It is either used to regenerate SAM, in which case it is initially remethylated to methionine by the vitamin-B12-dependent enzyme methionine synthase, using 5-methyl tetrahydrofolate as the methyl

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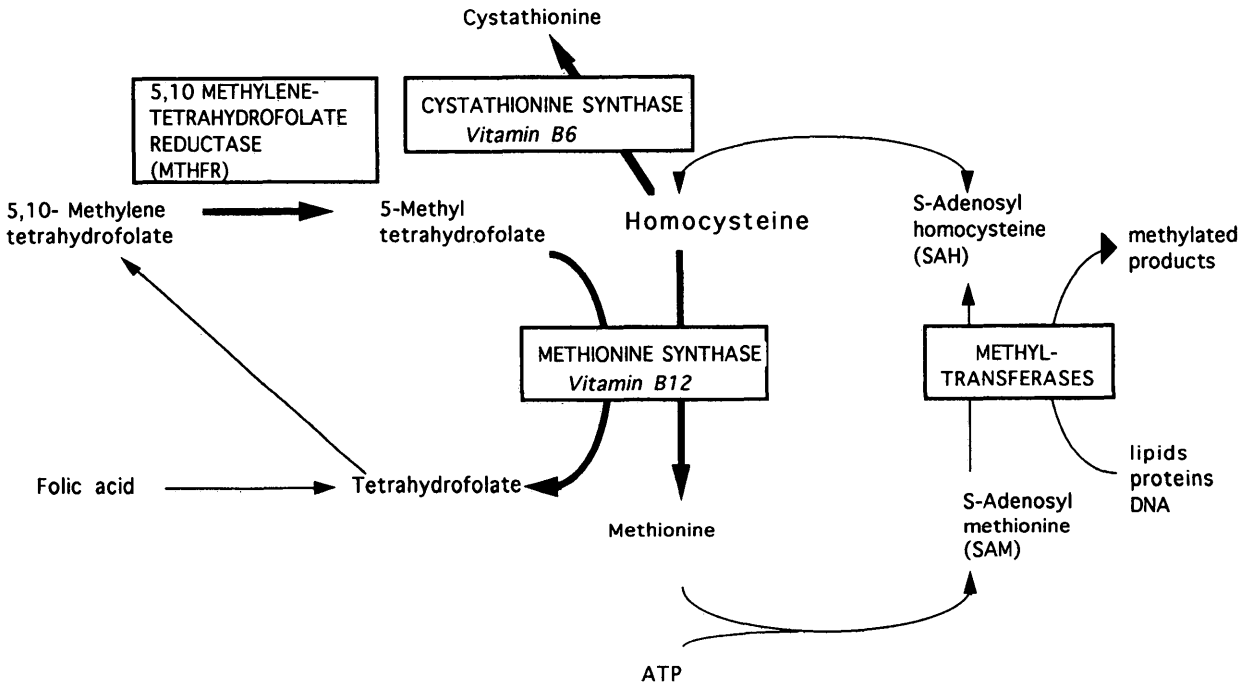

Figure 1. The production of homocysteine in methionine metabolism and the involvement of MTHFR. Arrows in bold represent points at which genetic defects in the enzyme or in co-factor biosynthesis are known to cause elevated homocysteine.

donor, or disposed of by the transsulphuration pathway in which the initial step is its condensation with serine to form the thioether cystathionine through the action of the vitamin-B6-dependent enzyme cystathionine-β-synthase (CBS) (Figure 1).

Of the enzymes involved in recycling or removing homocysteine, severe genetic defects resulting in loss of function are known to affect two: 5,10-methylene tetrahydrofolate reductase (MTHFR) and cystathionine-β-synthase (CBS). The genes encoding each of these two enzymes have been cloned and sequenced, and several mutations which cause such highly deficient phenotypes have been identified. Homozygotes for severe mutations of either the CBS or MTHFR genes have homocystinuria with associated premature vascular disease and thromboembolism affecting both large and small arteries and veins.[9,10] Heterozygotes for such severe mutations have mild hyperhomocysteinaemia; however, these genotypes are too rare to account for the frequency of mild hyperhomocysteinaemia observed in the general population.

MTHFR is a flavoprotein which catalyses the NADPH-linked reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the major circulating form of folate and the methyl donor for the methioninesynthase-catalysed remethylation of homocysteine to methionine. A phenotypic variant of MTHFR with characteristic thermolability after partial denaturation at 46°C for 5 min[11] has been identified in 5–8% of the healthy population and shown to give rise to mild hyperhomocysteinaemia without homocystinuria in healthy controls as well as CAD patients, in whom it is approximately three times more common than in healthy controls.[12–14] This biochemically-defined thermolabile variant is the most probable cause of mild hyperhomocysteinaemia in 28% of hyperhomocysteinaemic vascular disease patients.[15]

The cDNA for MTHFR was recently cloned and sequenced by Goyette *et al.*, who identified nine mutations in classically deficient patients.[16,17] In addition, a C to T transition at nucleotide 677, resulting in an amino acid change from alanine to valine, correlated with thermolability; homozygotes for the mutation had thermolabile MTHFR (defined by a specific activity of 50% of the normal mean, and residual activity after heat inactivation of $<36\%$ of the initial activity), while heterozygotes had intermediate thermolability.[18] The mutation resulted in elevated homocysteine levels irrespective of whether measurements were made after fasting or methionine loading. This mutation has subsequently been shown to confer a 2.9-fold risk of cardiovascular disease in Ireland (Gallagher *et al.*, submitted) and a 3.1-fold risk of cardiovascular disease in Holland[19]—figures which are similar to the relative risk associated with elevated homocysteine levels.

The frequency of homozygosity for thermolabile MTHFR in individuals with NTDs is approximately three times the average in the population,[20,21] and in Ireland 13% of NTDs may be directly attributed to this factor.[20]

Elevated homocysteine is clearly an important risk indicator for a range of clinical conditions. The magnitude of risk conferred by the thermolabile MTHFR genotype is similar to that associated with elevated homocysteine. To permit a rational assessment of the potential for screening the population to identify individuals genetically at risk of homocysteine-associated disease, and to devise appropriate treatments with homocysteine-lowering supplements such as folic acid, the proportion of individuals who are hyperhomocysteinaemic due to the thermolabile MTHFR genotype needs to be determined.

Our study examines the extent to which mild hyperhomocysteinaemia in a working male population is directly attributable to the thermolabile MTHFR mutation.

Methods

Males aged 30–49 from an industrial workforce in Belfast, comprising both manual and non-manual workers, were invited to a screening clinic, and after informed consent for all biochemical and genetic analyses had been given, a venous blood sample was taken with minimum haemostasis and anticoagulated with EDTA. Individuals who were diabetic, had had a general anaesthetic within the previous 3 months, or were using any form of dietary supplementation (16.6%) were excluded. A total of 625 men were eligible for inclusion in the present analysis.

Total homocysteine (free plus protein-bound) was assayed by high performance liquid chromatography according to the method of Araki & Sako,[22] modified by Ubbink *et al.*[23] Plasma samples were derivatized with ammonium 7-fluoro 2-oxa-1,3 diazole-4-sulphonate (SBD-F).

Genotyping for the MTHFR thermolabile mutation was performed by PCR and *Hin*fI digestion as in Frosst *et al.*[18]

Levels of serum folate and vitamin B12 were measured using an ICN Pharmaceuticals kit.

Statistics were analysed using SPSS for Windows. Distributions of homocysteine, folate and B12 were skewed and were logarithmically transformed when appropriate. Analysis of variance was used to compare mean values between groups (genotypes or tenths). Relative risk estimates were calculated using χ^2 tests with Yates' correction or Fisher's exact probability test as appropriate.

Results

In our study population, the overall frequencies of thermolabile homozygotes, heterozygotes, and non-thermolabile homozygotes were 11.5, 43.7, and 44.8% respectively (in close agreement with the Hardy-Weinberg prediction of 11.1, 44.5, and 44.4%: χ^2 test $p>0.5$).

Thermolabile homozygous individuals had mean homocysteine levels significantly higher than those of heterozygotes, whose homocysteine levels were slightly, but not significantly, higher than those of non-thermolabile homozygotes (Table 1). To assess the genotype/phenotype relationship we divided the population into tenths according to homocysteine ranking (Table 2). The thermolabile homozygote frequency exhibited a gradual increase from 1.6% in

Table 1 Plasma homocysteine, serum folate and vitamin B12 levels by MTHFR genotype

Genotype	+/+ (*n*=72)	+/− (*n*=273)	−/− (*n*=280)	Significance
Mean homocysteine (μmol/l)	9.46(8.40,10.65)	7.12(6.85,7.40)	6.77(6.55,7.01)	*
Mean homocysteine in men with folate below median (μmol/l)	11.22(9.57,13.16) (*n*=46)	7.88(7.47,8.31) (*n*=134)	7.43(7.02,7.86) (*n*=118)	*
Mean homocysteine in men with folate above median (μmol/l)	6.82(6.21,7.49) (*n*=24)	6.54(6.23,6.87) (*n*=133)	6.32(6.08,6.57) (*n*=156)	
Mean serum folate (nmol/l)	9.29(8.33,10.35)	10.76(10.26,11.29)	12.31(11.71,12.93)	**
Mean vitamin B12 (pmol/l)	221(198,248)	259(247,272)	266(253,279)	*

Geometric mean values of homocysteine, folate, and vitamin B12 are shown with 95% CIs (in brackets) for thermolabile homozygotes (+/+), heterozygotes (+/−), and non-thermolabile homozygotes (−/−). Mean homocysteine levels for men whose serum folate levels are above and below the median value (11.04 nmol/l) show that the association of high homocysteine with the +/+ genotype is highly dependent on folate status. * Significant difference ($p<0.003$) between the +/+ genotype and the other two genotypes; ** All three genotypes are significantly different from each other ($p<0.001$). Note: folate levels measured for 611 subjects.

Table 2 MTHFR genotypes in relation to homocysteine concentration

Homocysteine ranking by tenth		1	2	3	4	5	6	7	8	9	10
Percentage of each genotype within each tenth	+/+	35.5	11.3	18.8	8.2	6.3	9.5	11.3	6.3	6.3	1.6
	(*n*)	(22)	(7)	(12)	(5)	(4)	(6)	(7)	(4)	(4)	(1)
	+/−	40.3	43.5	50.0	50.8	50.8	31.7	40.3	41.3	42.9	45.2
	(*n*)	(25)	(27)	(32)	(31)	(32)	(20)	(25)	(26)	(27)	(28)
	−/−	24.2	45.2	31.3	41.0	42.9	58.7	48.4	52.4	50.8	53.2
	(*n*)	(15)	(28)	(20)	(25)	(27)	(37)	(30)	(33)	(32)	(33)

Individuals were assigned to tenths according to their homocysteine ranking: tenth 1 corresponds to highest homocysteine levels. The percentage of individuals within each tenth who are thermolabile homozygotes (+/+), heterozygotes (+/−), and non-thermolabile homozygotes (−/−) is shown.

the lowest tenth, to between 10% and 20% in the second and third highest tenths. In the upper tenth, the percentage of homozygotes rose appreciably to 35.5%, but there was a striking further increase in the frequency of thermolabile homozygotes among the individuals with the highest homocysteine rankings within this group. We therefore sequentially subdivided the upper tenth, and observed progressive increases in thermolabile homozygote frequency as the numbers of individuals were progressively restricted according to their ranking, i.e. 11 of the top 20 (55%), and 7 of the top 10 (70%). We calculated the relative risk of being in the top 5, 10, 20 and 50% of the population with respect to homocysteine for individuals with the thermolabile homozygous genotype relative to non-thermolabile homozygotes and to non-thermolabile homozygotes and heterozygotes combined (Table 3). There was a highly significant 9.7-fold risk of being in the top 5% for thermolabile homozygotes relative to non-thermolabile homozygotes. This risk was slightly less (7.2-fold) when thermolabile homozygotes were compared with non-thermolabile homozygotes and heterozygotes together. A small risk was observed for heterozygotes relative to non-thermolabile homozygotes, but this was only statistically significant in the upper 50% of the homocysteine distribution considered as a whole. Overall the data point to a small but significant thermolabile heterozygote effect in elevating homocysteine, which has not been reported previously.

When serum folate status was taken into account, the relationship between the homozygous thermolabile genotype and homocysteine levels was restricted to those individuals whose serum folate levels were below the median value ($p<0.001$). In individuals with folate levels above the median value, homocysteine levels did not vary significantly between genotypes ($p=0.3$) (Table 1).

Serum folate and vitamin B12 levels were both inversely related to homocysteine levels (Figure 2), and also correlated with each other (not shown). We observed a relationship between serum folate concentration and MTHFR genotype with homozygous thermolabile individuals having the lowest serum folate levels and non-thermolabile homozygotes the highest (Table 1). Heterozygotes had intermediate serum folate levels. The differences in folate levels between all three genotypes were significant ($p<0.001$). Vitamin B12 levels were also significantly lower in thermolabile homozygotes than in individuals with other genotypes (Table 1). The differences in folate and B12 levels between genotypes for each adjacent tenth were not statistically significant due to the small numbers of thermolabile homozygotes involved, but the trend is apparent,

Table 3 The relative risk of mild hyperhomocysteinaemia conferred by MTHFR thermolabile genotypes

Homocysteine rank	+/+ relative to −/−			+/+ relative to (+/− and −/−)			+/− relative to −/−		
	Risk	95% CI	*p*	Risk	95% CI	*p*	Risk	95% CI	*p*
Top 5%	9.72	(3.91,24.17)	<0.001	7.20	(3.72,13.93)	<0.001	1.71	(0.63,4.64)	0.29
Top 10%	5.70	(3.12,10.42)	<0.001	4.22	(2.67,6.88)	<0.001	1.71	(0.92,3.17)	0.08
Top 20%	2.62	(1.77,3.89)	<0.001	2.34	(1.68,3.28)	<0.001	1.24	(0.86,1.79)	0.25
Top 50%	1.69	(1.37,2.08)	<0.001	1.47	(1.23,1.75)	<0.001	1.31	(1.10,1.57)	0.003

Relative risks of being in the top 5, 10, 20 and 50% of the homocysteine distribution for individuals with the thermolabile homozygous genotype (+/+) relative to non-thermolabile homozygotes (−/−), and to non-thermolabile homozygotes and heterozygotes combined, and for thermolabile heterozygotes (+/−) relative to non-thermolabile homozygotes.

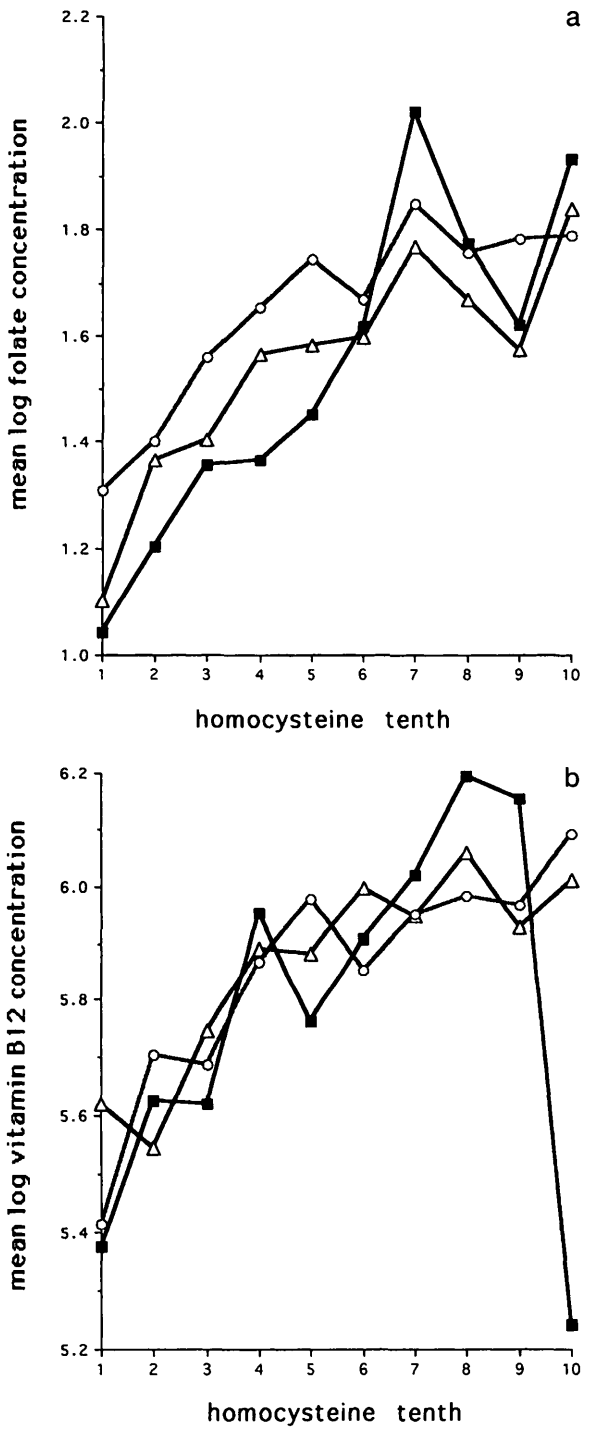

Figure 2. The relationship between **a** serum folate and **b** vitamin B12 levels and homocysteine. Mean log levels are plotted separately for each genotype within the homocysteine distribution (homocysteine decreases from tenth 1 to 10). Thermolabile homozygotes, filled squares; heterozygotes, open triangles; non-thermolabile homozygotes, open circles. The low B12 value for the homozygous thermolabile genotype in tenth 10 is due to the presence of only one such individual in this tenth.

particularly for serum folate in individuals in the upper half of the homocysteine distribution (Figure 2**a**).

We further examined the serum folate and vitamin B12 levels in the 20 individuals with the highest homocysteine levels. Most of these individuals had folate and B12 levels at the low end of the overall range. Consistent with the genotype/folate interaction already discussed, on average the thermolabile homozygotes had lower folate levels (mean 5.45 nmol/l, $n=11$), than individuals with the other two genotypes (combined mean 7.71 nmol/l, $n=9$). Vitamin B12 levels showed a similar trend. Some individuals in the top 20 are not thermolabile homozygotes and do not have marked deficiency of folate or vitamin B12, suggesting the existence of other, possibly genetic, factors influencing homocysteine levels.

Discussion

We have shown that in a working male population homozygosity for the thermolabile MTHFR genotype is a major contributing factor to mild hyperhomocysteinaemia, and in particular to homocysteine levels above the 95th percentile. The approximately fivefold increase in frequency of the thermolabile genotype in the top 5% of the population (to 48%) relative to its frequency in the bottom 80% is of particular note because one of the largest studies of the association of homocysteine with myocardial infarction (MI)—a prospective study of 14 916 physicians of whom 271 developed MI—suggested that the 95th percentile of homocysteine distribution was the point at which the risk of MI increased approximately three-fold.[1]

Dietary deficiency of the vitamin cofactors B6 (for CBS) and B12 (for methionine synthase), and of folic acid, the precursor of the methyl donor 5-methyltetrahydrofolate, can all cause elevated homocysteine levels, although evidence that this is a major contributing factor to hyperhomocysteinaemia only exists for an elderly population studied by Selhub *et al.*[24] (who estimated that up to 2/3 of hyperhomocysteinaemic 67–96 year olds from the Framingham Heart Study had elevated homocysteine attributable to deficiency of one of these three dietary components).

Our study identifies two separate aspects of the interaction between the homozygous thermolabile MTHFR genotype and folate metabolism.

Firstly, in individuals with serum folate levels below the median, those with the homozygous thermolabile genotype have higher homocysteine levels (50% higher than non-thermolabile homozygotes). Heterozygotes show a smaller increase (6%) in homocysteine relative to individuals with no thermolabile allele. Among individuals whose folate levels are above the median, the homozygous ther-

molabile genotype does not appear to affect homocysteine levels. Jacques *et al.* recently observed a similar effect in thermolabile homozygotes (but not heterozygotes) with folate levels below the median.[25] The folate level below which the thermolabile homozygous genotype becomes a homocysteine-determining factor has yet to be established. However it is apparent that low folate has a particularly detrimental effect on the capacity of thermolabile homozygotes to remethylate homocysteine.

Secondly, the thermolabile homozygotes as a group have significantly lower serum folate levels than the other genotypes, indicating that the thermolabile homozygous genotype itself causes reduced serum folate levels. It seems likely that the above two interactions between genotype and folate can act in concert to produce the mild hyperhomocysteinaemic phenotype.

The apparently causal relationship between genotype and serum folate levels indicates that in a proportion of individuals in the higher homocysteine range the lower folate levels are not necessarily attributable to dietary insufficiency alone but are, at least in part, a direct result of the reduced activity of the thermolabile enzyme. In a recent study of women with multiple NTD events, Lucock *et al.*[26] have proposed that the low levels of plasma 5-methyltetrahydrofolate (measured specifically by HPLC) relative to dietary folate intake observed in such cases, may reflect an underlying control or structural defect in the MTHFR gene or in another gene involved in an earlier step in the conversion of dietary folate to 5-methyltetrahydrofolate. They speculated that such cases may need a higher intake of dietary folate (or supplements) to achieve the same plasma 5-methyltetrahydrofolate concentrations as controls. In a subsequent study decreased serum folate levels and elevated red-cell folate levels were observed in thermolabile homozygotes by van der Put *et al.*[21] who point out that the MTHFR enzyme product 5-methyltetrahydrofolate is the predominant form of folate in serum, while other folate species including the substrate 5,10-methylenetetrahydrofolate are mainly present in cells.

In conclusion, it appears that homozygosity for the MTHFR thermolabile mutation is a major cause of mild hyperhomocysteinaemia in the study population. The frequency of the mutation may be expected to vary between populations, and this may account for some of the variation in homocysteine levels observed in different countries and regions (Malinow *et al.*, submitted). The extent to which this is due to variations in MTHFR genotype frequencies will not become clear until significant numbers of individuals from defined populations have been studied.

Dietary supplementation with folic acid given periconceptionally has already been shown to be effective in the reduction of NTDs,[27,28] some of which are caused by the thermolabile MTHFR allele.[20,21] Studies of dietary folic acid supplementation of healthy individuals and vascular disease patients indicate that this is a safe and effective method of reducing homocysteine levels (provided B12 is not deficient).[29–31] It is likely that extra dietary folic acid can compensate for the suboptimal function of the thermolabile form of MTHFR and can therefore be used to reduce the high homocysteine levels observed in many thermolabile homozygotes, thus eliminating this major risk factor for vascular and thrombotic disease. Our results establish that for a given homocysteine concentration thermolabile MTHFR homozygotes have lower serum folate levels, indicating that there is a direct genotype–folate interaction. We suggest that the thermolabile MTHFR genotype should be taken into account in the design of studies aiming to identify the optimum dose of folic acid required to lower homocysteine concentration, as the effectiveness of folate supplementation is likely to vary with genotype.

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