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Methionine Synthase D919G Polymorphism Is a Significant But Modest Determinant of Circulating Homocysteine Concentrations

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Elevation in plasma homocysteine concentration has been associated with vascular disease and neural tube defects. Methionine synthase is a vitamin B_{12}-dependent enzyme that catalyses the remethylation of homocysteine to methionine. Therefore, defects in this enzyme may result in elevated homocysteine levels. One relatively common polymorphism in the methionine synthase gene (D919G) is an A to G transition at bp 2,756, which converts an aspartic acid residue believed to be part of a helix involved in co-factor binding to a glycine. We have

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investigated the effect of this polymorphism on plasma homocysteine levels in a working male population (n = 607) in which we previously described the relationship of the C677T "thermolabile" methylenetetrahydrofolate reductase (MTHFR) polymorphism with homocysteine levels. We found that the methionine synthase D919G polymorphism is significantly (P = 0.03) associated with homocysteine concentration, and the DD genotype contributes to a moderate increase in homocysteine levels across the homocysteine distribution (OR = 1.58, DD genotype in the upper half of the homocysteine distribution, P = 0.006). Unlike thermolabile MTHFR, the homocysteine-elevating effects of the methionine synthase polymorphism are independent of folate and B_{12} levels; however, the DD genotype has a larger homocysteine-elevating effect in individuals with low B_6 levels. This polymorphism may, therefore, make a moderate, but significant, contribution to clinical conditions that are associated with elevated homocysteine. Genet. Epidemiol. 17:298–309, 1999.

Key words: folate; vitamin B_6; vitamin B_{12}; cardiovascular disease; neural tube defects

INTRODUCTION

Elevated levels of plasma homocysteine are being intensively investigated as a risk factor for vascular disease [Omenn et al., 1998; Welch and Loscalzo, 1998]. They are estimated to account for 10% of all coronary artery disease, and to contribute significantly to both cerebral and peripheral vascular disease [Boushey et al., 1995]. Hyperhomocysteinemia has also been observed in women with a history of pregnancies that result in neural tube defects (NTDs), and in those who subsequently give birth to children with NTDs [Steegers-Theunissen et al., 1994; Mills et al., 1995].

Homocysteine is an intermediate product of transmethylation reactions that consume S-adenosyl methionine, and may either be removed by cystathionine β-synthase (CBS) mediated transsulfuration, or remethylated to methionine by methionine synthase using 5-methyltetrahydrofolate as the methyl donor. CBS requires vitamin B_6 as a co-factor, while methionine synthase requires vitamin B_{12}. Deficiencies of vitamin B_6, B_{12}, or folate can, therefore, lead to elevated homocysteine levels, as can genetic defects in enzymes in the pathway. Severe enzyme defects give rise to homocystinuria, which is characterised by very high homocysteine concentrations, and an associated clinical syndrome including severe premature vascular disease. However, common enzyme variants that influence homocysteine levels within the normal range have only been found in one enzyme, 5,10-methylenetetrahydrofolate reductase (MTHFR), which catalyses the conversion of 5,10 methylenetetrahydrofolate to 5-methyltetrahydrofolate. The "thermolabile" variant of MTHFR results from a C to T transition at nucleotide 677 (C677T) [Frosst et al., 1995] and individuals homozygous for the T allele have significantly elevated plasma homocysteine levels [Harmon et al., 1996]. A second polymorphism in MTHFR, A1298C, which never occurs in cis with the C677T mutation, was recently identified and found to contribute to elevated homocysteine levels in individuals heterozygous for both mutations [van der Put et al., 1998].

The cDNA sequence of human methionine synthase was defined relatively recently [Leclerc et al., 1996; Li et al., 1996; Chen et al., 1997] and has been analysed for common polymorphisms that may influence homocysteine levels, and perhaps

contribute to vascular disease and NTD risk. Only one such candidate polymorphism has been identified so far. This is an A to G transition at nucleotide 2,756 in the open reading frame, which results in substitution of glycine for aspartic acid at amino acid position 919 (D919G) [Leclerc et al., 1996]. The G allele of this polymorphism has a frequency of 0.15–0.2 in cohorts studied so far [Leclerc et al., 1996; Chen et al., 1997; Morrison et al., 1997; van der Put et al., 1997]. In two small studies, the D919G enzyme variant was not associated with NTDs [Morrison et al., 1997; van der Put et al., 1997], and no significant association between D919G genotypes and plasma homocysteine levels has been found [van der Put et al., 1997]. However, these studies were relatively small with only sufficient statistical power to detect large effects.

We have analysed the relationship between D919G genotype and homocysteine concentration in a cohort of 625 working men aged 30–49, and present evidence that the DD genotype is associated with a modest increase in plasma homocysteine. In a previous study, we had shown the contribution of the C677T MTHFR polymorphism to elevated plasma homocysteine in this same cohort [Harmon et al., 1996], and could therefore assess whether the D919G genotype interacts with the MTHFR C677T genotype, serum folate, vitamins B_{12} or B_6 to modulate plasma homocysteine concentrations.

METHODS

The study cohort has been described previously [Harmon et al., 1996]. Briefly, males aged 30–49, comprising manual and non-manual workers from an industrial workforce, were invited to a screening clinic, and after informed consent for all biochemical and genetic analyses had been given, a venous blood sample was obtained. Individuals who were diabetic, had had a general anaesthetic within the previous 3 months, or who were using dietary supplementation, were excluded. A total of 625 men were eligible for the study.

Genotyping for the methionine synthase D919G mutation was as follows. PCR amplification was carried out using an exonic forward primer (5′-TGT TCC CAG CTG TTA GAT GAA AAT C-3′) and an intronic reverse primer (5′-GAT CCA AAG CCT TTT ACA CTC CTC-3′) to amplify a 211-bp fragment spanning the polymorphism. The polymorphism introduces an Hae III site in the rarer G allele, resulting in fragments of 80 and 131 bp. PCR products were digested with Hae III for 2 hr, followed by electrophoresis on 3% agarose gels and visualisation by ethidium bromide staining. Methionine synthase genotypes were obtained for 607 men.

Homocysteine, vitamins B_{12} and B_6, and MTHFR thermolabile genotype were obtained as described [Harmon et al., 1996; Woodside et al., 1997].

Statistical analysis was carried out using the STATA general statistics package [Statacorp, 1997]. Distributions of homocysteine, folate, and vitamins B_{12} and B_6 were all skewed, with a number of high values. Log-transformation removed most of this skew, but the resulting distributions were not normal, and for this reason ranked analysis was favoured. Distributions were displayed by presenting values in each decile, which eases comparison with previous analyses of the same dataset [Harmon et al., 1996]. To look at the interactions of the D919G genotype with folate, B_{12} and B_6 in their effect on homocysteine, analysis of variance of ranked values was chosen, which allows an assessment of interaction effects over and above main effects within the model. Distributions of homocysteine, folate, B_{12} and B_6 were converted

to ranks before analysis of variance. Interaction terms were fitted within analysis of variance models including their component terms. Mean homocysteine levels and confidence intervals were calculated on log-transformed values and the antilogarithm taken to return them to the usual scale of homocysteine. Relative risks were estimated by calculating the Odds Ratio (OR).

RESULTS

Relationship Between Methionine Synthase D919G Genotype and Homocysteine Level

Methionine synthase D919G genotype and homocysteine levels were obtained for 607 men. The G allele was present at a frequency of 20.5% in accordance with frequencies obtained in other populations [Leclerc et al., 1996; Chen et al., 1997; Morrison et al., 1997; van der Put et al., 1997]. The frequencies of GG homozygotes, DG heterozygotes, and DD homozygotes were 3.0, 35.1, and 61.9%, respectively (in close agreement with the Hardy-Weinberg prediction of 4.2, 32.6, and 63.2%, respectively). When the population was divided into tenths according to homocysteine concentration, there was a clear increase in DD genotype frequency as homocysteine level increased (Table I). To assess the significance of this effect, homocysteine values were ranked within the entire dataset in order to adjust for non-normal distribution. Analysis of variance detected a significant influence of methionine synthase genotype on homocysteine rank ($P = 0.03$). When the genotypic effect was modelled as the difference between DD and the other two genotypes, a similarly significant effect was obtained ($P = 0.01$), whereas the rare GG genotype analysed as a subgroup did not confer a significant effect ($P = 0.83$). A much larger study would be required to have sufficient power to assess the effect, if any, of this genotype.

Table I indicates that the DD genotype is at a higher frequency in the upper half of the homocysteine distribution and at a lower frequency in the lower half. In particular, the 20% of the population with the lowest plasma homocysteine concentrations has the lowest frequency of the DD genotype. To give an indication of the risk conferred, Table II shows the relative risk when comparing genotype frequencies across various homocysteine cut-off points. Regardless of which cut-off is chosen, between the top 10 and 80% of the distribution, a relative risk of around 1.5 is observed. The DD genotype, therefore, contributes to a modest increase in plasma homocysteine concentration across the range of homocysteine values. This contrasts with the homozygous thermolabile MTHFR genotype, which confers a greatly increased risk of having a homocysteine concentration that falls within the top 10% of the range.

Interactions of D919G Genotype and Homocysteine With Folate, Vitamins B_{12} and B_6

We have previously shown that homocysteine levels are inversely related to levels of serum folate and vitamin B_{12}, but show no correlation with vitamin B_6 concentration [Harmon et al., 1996; Woodside et al., 1997].

The current study indicates that the methionine synthase DD and DG genotypes are not differentially associated with folate levels (analysis of variance of folate rank, $P = 0.95$; Fig. 1a). In addition, they do not modify the correlation between folate and

TABLE I. Distribution of D919G Genotypes in the Population Ranked According to Homocysteine*

	Homocysteine ranking by tenth									
	1	2	3	4	5	6	7	8	9	10
Percentage of each genotype by tenth (n)										
GG	0	4.8	3.3	6.7	1.7	3.3	1.6	0	4.8	3.3
	(0)	(3)	(2)	(4)	(1)	(2)	(1)	(0)	(3)	(2)
GD	30.5	35.5	23.0	23.3	35.0	39.3	36.1	38.3	45.9	44.3
	(18)	(22)	(14)	(14)	(21)	(24)	(22)	(23)	(28)	(27)
DD	69.5	59.7	73.8	70.0	63.3	57.4	62.3	61.7	50.8	52.5
	(41)	(37)	(45)	(42)	(38)	(35)	(38)	(37)	(31)	(32)
Mean Hcy	14.13	9.55	8.71	7.76	7.24	6.76	6.31	5.89	5.25	4.17
Min Hcy	10.23	9.12	8.32	7.59	7.08	6.61	6.03	5.62	4.90	1.62
Max Hcy	77.62	10.23	9.12	8.13	7.59	7.08	6.61	6.03	5.62	4.90

*Individuals were assigned to tenths according to their homocysteine ranking: tenth 1 corresponds to highest homocysteine. Shown are the percentage of individuals within each tenth who have the GG, DG, or DD genotypes, and the number of subjects in parentheses. The mean, minimum (min), and maximum (max) homocysteine (Hcy) concentrations (μmol/l) are shown for each tenth.

TABLE II. Relative Risk of Elevated Homocysteine Associated With the DD Genotype at Different Homocysteine Cut-Off Levels*

	DD vs. DG and GG					
	All subjects (n = 607)			Lowest quartile vitamin B6 (n = 138)		
Homocysteine rank (%)	Rel. risk	95% CI	*P*	Rel. risk	95% CI	*P*
Top 10	1.48	0.84–2.63	0.178	0.80	0.25–2.55	0.71
Top 40	1.59	1.13–2.23	0.007	2.50	1.21–5.15	0.01
Top 50	1.58	1.14–2.19	0.006	2.57	1.28–5.15	0.01
Top 80	1.72	1.66–2.55	0.007	2.54	1.04–6.15	0.04

*Relative risks of being in the top 10, 40, 50, and 80% of the homocysteine distribution for individuals with the methionine synthase DD genotype relative to other genotypes (combined) for the whole cohort and for individuals in the lowest quartile of the vitamine B6 distribution. B6 values and D919G genotype were obtained for 549 subjects.

homocysteine that is observed in the study population as a whole. Analysis of variance indicated that homocysteine rank was significantly influenced by methionine synthase genotype and folate rank, but a term added to test their interaction was not significant ($P = 0.49$). This is illustrated in Figure 1a.

Similarly, methionine synthase genotype does not affect the correlation between vitamin B_{12} and homocysteine. Analysis of variance indicated that homocysteine rank was influenced by methionine synthase genotype and B_{12} rank, but a term added to test their interaction was not significant ($P = 0.90$) (Fig. 1b). B_{12} rank was not significantly dependent on methionine synthase genotype ($P = 0.64$).

The 10 individuals in the top half of the homocysteine distribution who have the GG genotype appear to have lower folate and vitamin B_{12} levels, but their numbers are too small to reach significance for either nutrient.

Vitamin B_6 is not correlated with homocysteine in this dataset (Spearman rank correlation $P = 0.21$), nor is B_6 rank affected by methionine synthase genotype ($P = 0.94$, analysis of variance). Surprisingly, there appears to be an interaction of B_6 rank with methionine synthase genotype that has a significant effect on homocysteine levels ($P = 0.05$). Clearly, the significance of this finding must be assessed in the light of the multiple tests that have been applied as part of this exploratory analysis of folate, vitamin B_{12} and B_6 interactions and main effects. After multiple testing, this observation is no longer significant ($P = 0.30$). Nevertheless, it may provide a useful clue to the possible interaction of these factors. This observation reflects lower B_6 levels in DD than DG subjects at the upper end of the homocysteine distribution (Fig. 1c). Thus, for individuals in the lowest quartile of the B_6 distribution, the DD genotype confers a relative risk of 2.50 ($P = 0.01$) of falling in the top 40% of the homocysteine distribution (Table II).

Interactions of Methionine Synthase D919G With MTHFR Thermolabile Genotype

Both the methionine synthase DD genotype and the MTHFR thermolabile TT genotype are associated with elevated levels of homocysteine (Table III). The high risk combination TT/DD is associated with a higher homocysteine level than other genotype combinations, but not significantly more so than would be expected if the effects of the two genotypes are additive. It can be seen from Table III that, among

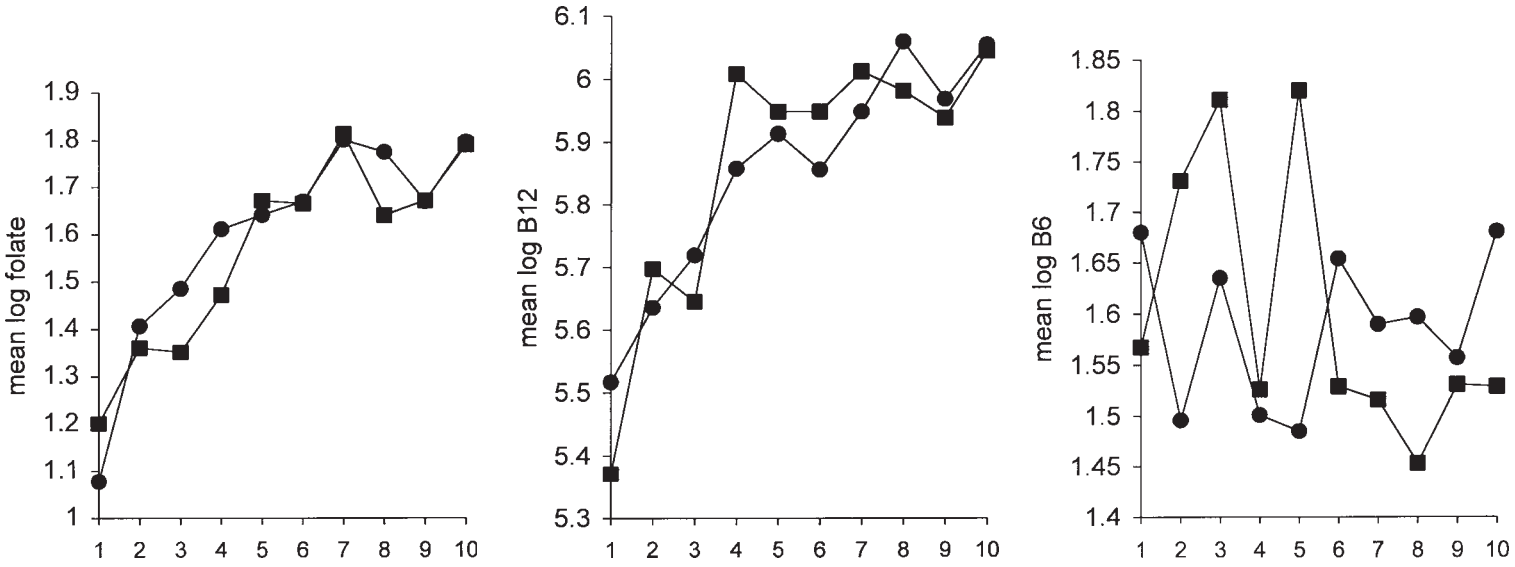

Fig. 1. Mean log folate **(a)**, B12 **(b)**, and B6 **(c)** are shown for each homocysteine tenth (tenth 1 corresponds to highest homocysteine). DD and DG genotypes are plotted separately. GG genotypes have been omitted because their numbers within each tenth are too few to give a meaningful result. Circles, DD; squares, DG.

TABLE III. Mean Log Adjusted Homocysteine Concentration for Each MTHFR/Methionine Synthase Genotype Combination[a]

	Methionine synthase genotype (n)			
MTHFR genotype	GG	DG	DD	All
CC	7.03 (6)	6.52 (101)	6.89 (1.65)	6.74 (272)
	[5.4–9.1]	[6.2–6.9]	[6.6–7.2]	[6.5–7.0]
CT	6.81 (8)	6.92 (88)	7.26 (169)	7.13 (265)
	[5.1–8.9]	[6.5–7.4]	[6.9–7.6]	[6.9–7.4]
TT	7.64 (4)	8.99 (24)	9.79 (42)	9.37 (70)
	[6.3–9.1]	[7.1–11.5}	[8.5–11.2]	[8.3–10.5]
All	7.06 (18)	6.91 (213)	7.35 (376)	
	[6.2–8.0]	[6.6–7.3]	[7.1–7.6]	

[a]Mean homocysteine levels are given in μmol/l. Means and 95% confidence intervals (in brackets) were calculated on log homocysteine values, and then converted to the ordinary scale. The number of subjects with each genotype combination is given in parentheses.

TT subjects, the ranges of the DD and DG genotypes overlap considerably, indicating that there is unlikely to be any significant interaction. Analysis of variance confirmed that homocysteine levels are not significantly influenced by the interaction between MTHFR and methionine synthase genotypes. The absence of clear synergy between methionine synthase and MTHFR genotypes suggests that they act independently on homocysteine concentrations.

Relative Contributions of Genotypes and Vitamin Levels to Homocysteine Elevation

We have quantified the relative contributions of the methionine synthase and MTHFR genotypes to homocysteine elevation. The adjusted R^2 value from the analysis of variance model indicates the proportion of variance in homocysteine rank that is attributable to various factors. Folate is the most important correlated parameter, accounting for 18.1% of the variation. Folate and B_{12} together account for 25% of the variation. The methionine synthase DD genotype accounts for 0.9% of the homocysteine variation, compared to the MTHFR TT genotype, which contributes four times as much (4.1%). While B_6 in itself makes no appreciable contribution (0.1%), methionine synthase DD, B_6, and their interaction accounted for 1.5% of homocysteine variation.

DISCUSSION

We have shown that the methionine synthase D919G polymorphism has a significant but moderate effect on homocysteine concentrations in a relatively large, working male population. The DD genotype is a determinant of elevated homocysteine, conferring a relative risk of 1.58 (CI 1.14–2.19) for having a homocysteine level in the top half of the distribution ($P = 0.006$). In contrast to the risk conferred by the homozygous thermolabile MTHFR genotype, which is most pronounced in the upper 10% of the homocysteine distribution (OR = 4.22 for TT homozygotes falling in the top 10%) [Harmon et al., 1996], the methionine synthase DD genotype confers a modest increase in risk (OR approximately 1.5 for individuals with the DD

genotype) that is not confined to one part of the homocysteine distribution. This appears consistent with our knowledge of the biochemistry underlying homocysteine metabolism. Methionine synthase acts directly to convert homocysteine to methionine, so an individual in whom the enzyme has reduced efficiency will tend to accumulate more homocysteine than an individual with a fully efficient enzyme, irrespective of the initial concentration of homocysteine. In contrast, MTHFR acts indirectly on homocysteine by generating 5-methyltetrahydrofolate (5-MTHF), the methyl group donor that is used by methionine synthase. Homocysteine clearance is compromised when concentrations of the methyl donor, 5-MTHF, which is the product of MTHFR acting on 5,10-methylenetetrahydrofolate, fall below a certain threshold. This is more likely to occur when an individual has the mildly dysfunctional thermolabile enzyme specified by the MTHFR TT genotype together with a low plasma folate level. Therefore, the effect of the MTHFR TT genotype is particularly pronounced in individuals who lie towards the top of the homocysteine range, who also tend to have lower folate levels.

The risk of elevated homocysteine that is conferred by the methionine synthase DD genotype is not greatly increased in men with low folate or vitamin B_{12} levels. This also is in stark contrast to the MTHFR TT genotype, which confers a much higher risk of elevated homocysteine in individuals with folate levels below the median level [Harmon et al., 1996; Jacques et al., 1996]. Methionine synthase is a vitamin B_{12}-dependent enzyme, and some interaction might have been expected between the genotype and vitamin B_{12} levels. However, this lack of association between low B_{12} levels and methionine synthase D919G is consistent with the location of this polymorphism in a domain of the protein that interacts with adenosylmethionine and auxiliary proteins. These are required for the reductive methylation and reactivation of the B_{12} cofactor, which can get inactivated by oxidation during catalysis. This domain is distinct from the B_{12} cofactor binding domain. It is tempting to speculate that the D allele might impair the binding of adenosylmethionine and/or auxiliary proteins, or possibly impair the stability of the protein. However, we and others have thus far been unable to express human methionine synthase at sufficient levels in an active form to evaluate the biochemical effects of this polymorphism.

Specific combinations of risk conferring MTHFR and methionine synthase genotypes do not confer significant additional risk over the sum of their individual effects. Thus, polymorphic variants of these 2 enzymes elevate homocysteine by compromising different parts of the pathway that do not directly interact with one another. While the homocysteine elevating effect of the thermolabile MTHFR enzyme depends on low folate levels, that of the methionine synthase D variant is folate and B_{12}-independent, but is enhanced in individuals with low vitamin B_6 levels. One possible explanation for this apparent interaction with B_6 concentration is that, as individuals with the DD genotype accumulate homocysteine due to a relatively inefficient enzyme, they rely more heavily on the B_6-dependent transsulfuration pathway to remove excess homocysteine.

Although the suggested influence of vitamin B_6 on the homocysteine-elevating effects of the DD genotype was not anticipated by us ab initio, it is of interest as recent studies have identified an association between low plasma B_6 levels and myocardial infarction [Chasan-Taber et al., 1996], vascular disease [Robinson et al., 1998], and coronary heart disease [Folsom et al., 1998]. One of these studies was retrospec-

tive [Robinson et al., 1998], and found in addition an association between CHD and elevated homocysteine; however, the relationship between B6 and atherosclerosis was independent of homocysteine. The other two studies were prospective [Chasan-Taber et al., 1996; Folsom et al., 1998], and like some other such studies, failed to find any evidence of an association between high homocysteine and vascular disease [Alfthan et al., 1994; Verhoef et al., 1994; Evans et al., 1997]. Other prospective studies have, however, shown an association of stroke and ischemic heart disease with elevated homocysteine [Arnesen et al., 1995; Perry et al., 1995; Wald et al., 1998]. Furthermore, homocysteine was prospectively shown to be strongly associated with mortality in individuals with angiographically confirmed coronary artery disease [Nygard et al., 1997]. The true nature of the relationship between homocysteine and the risk of developing vascular disease is, therefore, unclear given the present data. The evidence overall from prospective studies is that low vitamin B_6 levels may be a greater risk factor for vascular disease than homocysteine. The methionine synthase D919G polymorphism may constitute a genetic link between low vitamin B_6 status and elevated homocysteine, and may therefore partly explain some of the trends observed in the above-cited retrospective and prospective studies. We also note that in most of these studies, vitamin B_6 showed an inverse correlation with homocysteine as has been reported previously [Selhub et al., 1993], while there is no association of B_6 and homocysteine in our dataset; Verhoef and co-workers [1996] also did not observe any correlation between these variables.

To summarise, the methionine synthase DD genotype may, by contributing to elevated homocysteine, be a moderate risk factor for vascular disease and perhaps neural tube defects. This assumes that homocysteine is involved in the pathogenesis of these conditions, rather than being merely a marker for causative agents. Two studies in relatively small numbers of patients with NTDs [van der Put et al., 1997; Morrison et al., 1997] and patients with spiral arterial disease (a vascular disease of the placenta) [van der Put et al., 1997], have found no association of the methionine synthase D919G polymorphism with elevated homocysteine or disease. However, because of the very moderate effect of the mutation on homocysteine levels defined in this paper, much larger studies would be required to provide the statistical power to detect, or rule out, any association of this polymorphism with the above clinical conditions. For the same reason, future studies of the methionine synthase D919G polymorphism and its associations with atherothrombotic diseases should also be very large, and should include measurements of vitamin B_6, in addition to homocysteine, B_{12}, and folate.

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