Methionine synthase polymorphism A2756G is associated with susceptibility for thromboembolic events and altered B vitamin/thiol metabolism

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Background and Objectives. Vitamin B12 dependent methionine synthase (MS) regulates de novo production of methionine from homocysteine (Hcy). Since moderate elevations in Hcy are considered vasculotoxic, we examined a common variant (A2756G-MS) of the gene coding for this enzyme as a risk for thromboembolism.

Design and Methods. We investigated A2756G-MS and folate/thiol status in 51 individuals who had experienced a thromboembolic event (TE) and 95 subjects being treated for non-thromboembolic (NTE) vascular problems.

Results. The prevalence of the mutant G allele was lower in TE subjects than in controls, indicating a protective role for this base substitution (OR 0.39; 95%CI 0.20-0.78; p=0.010). Consistent with an advantage conferred by this allele, heterozygotes had generally lower levels of Hcy and glutathione (GSH), and higher levels of B-vitamins than wildtypes. The OR for the wildtype having an increased risk for TE was 2.32 (95%CI 1.06-5.08). Additionally, as might be predicted, TE-wildtypes had elevated GSH levels compared to corresponding NTE-wildtypes (p=0.004) - a likely response to oxidative stress. NTE subjects showed a dramatic reduction in Hcy between wildtype and heterozygote (p=0.017), and again between recessive and heterozygote genotypes (p=0.002). The same pattern, although not significant, occurred in TE subjects. The similarity in Hcy between clinical groups for each genotype raises questions on the etiological role of Hcy in TE. The functional relationship between enzyme variant and its B12-cofactor may be of more interest, since the polymorphic site occurs near the B12-binding domain, and our results indicate wildtype-TE subjects have a much lower level of vitamin B12 than heterozygote-TE subjects (p=0.0019). This effect is attenuated in NTE subjects.

Interpretation and Conclusions. A2756G-MS may protect against a thromboembolic event. The role of Hcy remains unclear.

Key words: folate, homocysteine, thromboembolism, A2756G methionine synthase, polymorphism.

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Thrombosis
research paper
haematologica 2002; 87:751-756
valve replacement). We present data on vitamin B12, erythrocyte folate, plasma folate, Hcy, cysteine (Cys) and an index of transsulphuration that is indicative of oxidative stress - glutathione (GSH). Redox changes associated with oxidative stress are of interest since they may increase the flux of Hcy through the transsulphuration pathway to Cys and GSH via a regulatory role at MS and cystathionine-β-synthase. This, it is claimed, may be a self-correcting response to depleted GSH in cells facing oxidative challenge. We were, therefore, particularly interested to see whether A2756G MS does reduce Hcy and offer protection against TE events, and in so doing, whether it is associated with lower GSH levels.

Design and Methods

Subjects
To clarify the association between A2756G MS G allele frequency and risk of a TE event, we examined 146 patients attending the local anticoagulant clinic. Samples were collected over 9 months and separated according to clinical history into those from individuals who had experienced a TE event (i.e. deep vein thrombosis) or those from patients who were being treated for NTE vascular problems such as valve replacement. These individuals were designated as case (n=51) or control (n=95) subjects, respectively. The median age and interquartile range (IQR) for TE and NTE subjects was 67.5 years (52-77) and 71 years (60-79), respectively. The difference in age between groups was not significant. TE subjects were 51% male, 49% female and NTE subjects 46% male, 54% female. Table 1 provides clinical data on each cohort.

Analysis
The assay for plasma thiols measures both oxidized and reduced forms. For example, total Hcy refers to homocysteine, homocystine, homocysteine-cysteine mixed disulphide and protein-bound forms of Hcy. The same holds true for analysis of total Cys and total GSH. Plasma total thiols (Hcy, Cys and GSH) were measured using isocratic HPLC following derivatization with the fluorogenic reagent SBDF. Plasma and red cell folate along with vitamin B12 were measured using a paramagnetic-particle, chemiluminescent immunoassay (Access Immunoassay System, Beckman Instruments, Inc).

A2756G MS genotyping was based on the method of Van der Put et al. Briefly, sense and antisense primers with the sequences 5’- GGT GTG TTC CCA GCT GTT AGA TG-3’ and 5’- GAC ACT GAA GAC CTC GAA C-3’ respectively, were used for PCR amplification. The amplicons were digested with the restriction enzyme HaeIII to yield a 265 bp fragment for the wildtype (AA), 265, 180 and 85 bp fragments for the heterozygote (AG), and 180 and 85 bp fragments for the homozygous recessive genotype (GG).

Statistics
The Anderson-Darling test was used to ascertain normality. Significant differences for unpaired data were then established using either an independent two sample t- or Mann-Whitney test. The degree and significance of an allele as a risk factor for TE was calculated using the Odds ratio with associated confidence intervals. The p value was obtained using Yates’ corrected χ² test.

Results
The frequency of AA, AG and GG genotypes was 78.4, 19.6 and 2.0%, respectively, for TE subjects and 61.1, 27.4 and 11.6%, respectively, for NTE individuals. Table 2 gives both allele number and allele frequency.

Table 1. Clinical background for TE and NTE cohorts.

<table>
<thead>
<tr>
<th>Event Type</th>
<th>Thromboembolic event</th>
<th>Non-thromboembolic event</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Pulmonary embolism</td>
<td>36.5%</td>
<td>62%</td>
</tr>
<tr>
<td>% Deep vein thrombosis</td>
<td>42%</td>
<td>31%</td>
</tr>
<tr>
<td>% Other (e.g., thrombotic cerebrovascular accident)</td>
<td>21.5%</td>
<td>7%</td>
</tr>
</tbody>
</table>

Table 2. Number and frequency of alleles for A2756G MS in 51 thromboembolic and 95 non-thromboembolic subjects.

<table>
<thead>
<tr>
<th>Subject group</th>
<th>A</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thromboembolic</td>
<td>90 (88.2)</td>
<td>12 (11.8)</td>
</tr>
<tr>
<td>Non-thromboembolic</td>
<td>142 (74.7)</td>
<td>48 (25.3)</td>
</tr>
</tbody>
</table>

GAC CTC TGA TTT GAA C-3' respectively, were used for PCR amplification. The amplicons were digested with the restriction enzyme HaeIII to yield a 265 bp fragment for the wildtype (AA), 265, 180 and 85 bp fragments for the heterozygote (AG), and 180 and 85 bp fragments for the homozygous recessive genotype (GG).

Statistics
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Results
The frequency of AA, AG and GG genotypes was 78.4, 19.6 and 2.0%, respectively, for TE subjects and 61.1, 27.4 and 11.6%, respectively, for NTE individuals. Table 2 gives both allele number and allele frequency.

An Odds ratio indicates that the G allele may afford significant protection against TE: OR = 0.39, 95% CI 0.20-0.78. χ² gives a p value of 0.0065 (Yates’ corrected p value = 0.01). Carriage of the G
A2756G methionine synthase and thromboembolism

A2756G methionine synthase and thromboembolism allele (AG + GG versus AA) also seemed to afford protection against TE: OR = 0.43, 95% CI 0.20-0.94. \( \chi^2 \) gave a p value of 0.0331 (Yates’ corrected p value = 0.05). When each genotype was individually compared to the wildtype, an OR consistent with a protective effect against TE was still observed, although significance was not quite achieved (OR = 0.13 and 0.56 for AA versus GG and AA versus AG, respectively).

Table 3 lists the median and interquartile range (IQR) for blood B vitamin and thiol levels between patients with the various MS genotypes in each clinical group, and indicates any statistical differences (both within and between clinical groups). A comparison of all TE and NTE subjects, independent of MS genotype, showed that only two parameters differed significantly; GSH was higher in TE individuals than in NTE ones (p = 0.0008), while its precursor Cys was lower in TE subjects than in those with NTE subjects (p = 0.0146). When examined on a genotype-dependent basis, three major statistical differences were detected, which in the first two cases, support the view that A2756G MS may exhibit a heterozygote advantage:

- significantly lower Hcy in NTE-AG patients compared to in NTE-AA ones; p = 0.0173 (also significantly lower Hcy in NTE-AG patients compared to in NTE-GG ones; p = 0.002);
- significantly higher vitamin \( B_{12} \) in TE-AG patients compared to in TE-AA ones; p = 0.0019;
- significantly higher GSH in TE-AA patients compared to in NTE-AA ones; p = 0.0035.

Although significance was not always attained, the overall trend for all measured parameters in Table 3 indicates that A2756G MS exhibits a heterozygote advantage. This is deduced because in both clinical groups the change between AA and AG genotypes was associated with an increase in serum folate, RBC folate and vitamin \( B_{12} \), and a concomitant decrease in Hcy, Cys and GSH. In other words, the heterozygote equilibrium would seem to be moving in the direction of increased MS turnover as opposed to Hcy build up or transsulphuration.

It is also worth mentioning that Cys levels in TE patients with AA and AG genotypes were lower than Cys levels in the corresponding NTE patients with AA and AG genotypes. This reduction approached significance in both cases - p = 0.0725 and 0.0582, respectively.

Discussion

The central role of folate metabolism in nucleotide and methionine biosynthesis means that common single nucleotide polymorphisms (SNPs) that influence 1C transfers can act as both a risk factor and defence against disease. For instance, C677T MTHFR is a risk factor for neural tube\(^7\) and other mid-line defects\(^8\), Down’s syndrome\(^9\) and complications of pregnancy such as pre-eclampsia\(^10, 11\) and recurrent early pregnancy loss.\(^12\) However, it can also, under certain circumstances, protect against colon cancer\(^13\) and acute lymphoblastic leukemia.\(^14\) The most likely explanation for this dichotomy of effect is that C677T MTHFR increases levels of 5,10-methylene-H\(_4\)folate, a critical coenzyme required for nucleotide biosynthesis (Figure 1). Under good folate nutrition, an accumulation of 5,10-methylene-H\(_4\)folate, a critical coenzyme required for nucleotide biosynthesis (Figure 1). Under good folate nutrition, an accumulation of 5,10-methylene-H\(_4\)folate, a critical coenzyme required for nucleotide biosynthesis (Figure 1). Under good folate nutrition, an accumulation of 5,10-methylene-H\(_4\)folate, a critical coenzyme required for nucleotide biosynthesis (Figure 1). Under good folate nutrition, an accumulation of 5,10-methylene-H\(_4\)folate, a critical coenzyme required for nucleotide biosynthesis (Figure 1). Under good folate nutrition, an accumulation of 5,10-methylene-H\(_4\)folate, a critical coenzyme required for nucleotide biosynthesis (Figure 1). Under good folate nutrition, an accumulation of 5,10-methylene-H\(_4\)folate, a critical coenzyme required for nucleotide biosynthesis (Figure 1). Under good folate nutrition, an accumulation of 5,10-methylene-H\(_4\)folate, a critical coenzyme required for nucleotide biosynthesis (Figure 1). Under good folate nutrition, an accumulation of 5,10-methylene-H\(_4\)folate, a critical coenzyme required for nucleotide biosynthesis (Figure 1). Under good folate nutrition, an accumulation of 5,10-methylene-H\(_4\)folate, a critical coenzyme required for nucleotide biosynthesis (Figure 1). Under good folate nutrition, an accumulation of 5,10-methylene-H\(_4\)folate, a critical coenzyme required for nucleotide biosynthesis (Figure 1). Under good folate nutrition, an accumulation of 5,10-methylene-H\(_4\)folate, a critical coenzyme required for nucleotide biosynthesis (Figure 1). Under good folate nutrition, an accumulation of 5,10-methylene-H\(_4\)folate, a critical coenzyme required for nucleotide biosynthesis (Figure 1). Under good folate nutrition, an accumulation of 5,10-methylene-H\(_4\)folate, a critical coenzyme required for nucleotide biosynthesis (Figure 1). Under good folate nutrition, an accumulation of 5,10-methylene-H\(_4\)folate, a critical coenzyme required for nucleotide biosynthesis (Figure 1). Under good folate nutrition, an accumulation of 5,10-methylene-H\(_4\)folate, a critical coenzyme required for nucleotide biosynthesis (Figure 1).
In the present study it appears that heterozygosity for A2756G MS is associated with the lowest Hcy (and Cys) levels as well as diminished GSH and enhanced vitamin B12 and folate status. These are all positive indices and are consistent with the significant finding that carriage of the G allele offers reduced risk of a thromboembolic event (OR 0.39). Thus, A2756G MS, like C677T MTHFR, may, under certain conditions, be another folate SNP with possible health benefits.

It is interesting to consider the role of MS in Hcy metabolism, and in particular the balance between Hcy remethylation, and transsulphuration to Cys and on to GSH (Figure 1). The data presented here are consistent with both clinical groups benefiting from heterozygosity for this SNP. Of course, it is equally clear that among individuals who do not carry a mutant allele, there is an increased risk of TE (OR = 2.32 95% CI 1.06-5.08). Indeed, compared to NTE-wildtypes, TE-wildtypes exhibit a significantly elevated level of GSH and a lower Cys (approaching significance). Hcy levels do not differ between these two categories. This would seem to suggest that although A2756G MS is capable of altering the turnover of Hcy at this enzyme locus, the effect does not obviously differ between clinical groups. This is despite more Cys apparently being used to enhance GSH levels in TE subjects than in NTE subjects. Therefore, the negative influence of carrying no mutant MS allele in TE subjects is most likely increasing GSH production as a mechanism to attenuate oxidative stress. However, while Hcy levels are generally higher in individuals with the wildtype MS enzyme than in heterozygotes, no significant difference exists between TE and NTE subjects. This suggests that Hcy may, in fact, not be a clinical factor in the causation of TE in these subjects. A similar conclusion regarding the vasculotoxic role of Hcy has been drawn by other researchers studying a link between vascular problems and the closely related polymorphic A66G methionine synthase reductase (MSR) gene.16

Although in general terms, mild hyperhomocys-
teinemia is now a well-established risk factor for a TE event. Others have also examined A2756G MS as a risk factor for TE: Salomon et al. concluded that A2756G MS was not a statistically significant risk factor for idiopathic venous thromboembolism. Despite this, and in accord with our data, Tsai et al. have recently shown that the A2756G transition is associated with lower Hcy levels, while Hynman et al. reported that heterozygosity for A2756G MS increases red cell folate and reduces secondary vascular events. The designs of these various studies differ. Our own findings may in part be a reflection of our control population, who are in themselves, a cohort of patients with vascular problems. Despite this, our study contributes further preliminary data indicating that the common A2756G polymorphism of MS may be an etiological factor in TE. However, like previous studies that implicate this SNP as a factor in myocardial infarction, larger studies would be desirable. Such studies might also be able to detect exactly how the biological function of MS is altered in this SNP. At this stage it is interesting to speculate that it may be linked to vitamin B12 metabolism in some way since the mutation occurs close to the B12 binding domain, and both clinical groups exhibit increasing plasma vitamin B12 levels with increasing carriage of the mutant allele.

In fact, in the at risk TE patients with a wildtype genotype, plasma vitamin B12 levels are significantly lower than in the TE-heterozygote genotype patients (p = 0.0019).

As interesting as these findings are, considerable work is required for a full elucidation of the complexity of all the possible interactions and clinical consequences involved. In particular, the potential existence of polymorphisms in the 3’ untranslated sequence of the MS gene requires further study, since such variants might influence the MS message and levels of the enzyme. The interaction (linkage disequilibrium) between such putative mutations, A2756G MS and three silent MS mutations (A2053T, A2127G and A3144G), as well as with related SNPs such as A66G MSR will, most likely, be of great interest in this particular area of clinical research in the future.

In summary, this study shows how folate, thiol and vitamin B12 metabolism are intimately linked at the level of this important genetic locus. It also illustrates that folate biochemistry (particularly genetic variation and nutrition) is a likely factor in vascular disease.

Contributions and Acknowledgments
ZY: planning, analysis, interpretation, formulation and critical revision of paper, approval of final manuscript; ML: conception and design of project, interpretation and analysis of data, formulation and critical revision of paper, particularly for intellectual content, approving final version of manuscript.

Disclosures
Conflict of interest: none.
Redundant publications: no substantial overlapping with previous papers.

Funding
This work was supported by the British Heart Foundation.

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**PEER REVIEW OUTCOMES**

**Manuscript processing**

This manuscript was peer-reviewed by two external referees and by Professor Vicente Vicente, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Prof. Vicente and the Editors. Manuscript received March 19, 2002; accepted May 23, 2002.

**What is already known on this topic**

It has been indicated that high plasma levels of homocysteine increase the risk of thromboembolic events. Methionine synthase polymorphism A2756G seems to attenuate homocysteine plasma levels.

**What this study adds**

The authors investigate the prevalence of this polymorphism and vitamin B12/thiol metabolism in patients who had experienced a thromboembolic episode and controls.

**Potential implications for clinical practice**

The results suggest that A2756G methionine synthase polymorphism may protect against thromboembolic events. However, the role that homocysteine plasma levels play in this association is not clear.

Vicente Vicente, Deputy Editor