High frequency of vitamin B12 deficiency in asymptomatic individuals homozygous to MTHFR $C677T$ mutation is associated with endothelial dysfunction and homocysteinemia

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Zittan E, Preis M, Asmir I, Cassel A, Lindenfeld N, Alroy S, Halon DA, Lewis BS, Shiran A, Schliamser JE, Flugelman MY. High frequency of vitamin B12 deficiency in asymptomatic individuals homozygous to MTHFR $C677T$ mutation is associated with endothelial dysfunction and homocysteinemia. Am J Physiol Heart Circ Physiol 293: H860–H865, 2007. First published May 4, 2007; doi:10.1152/ajpheart.01189.2006.—The aim of this study was to examine the association of homocysteinygosity for the methylenetetrahydrofolate reductase (MTHFR) $C677T$ mutation and vitamin B12 deficiency in 360 asymptomatic individuals and to investigate forearm endothelial function in $C677T$ homozygotes. MTHFR $C677T$ mutation and levels of vitamin B12, folate acid, and homocysteinemia were measured in study participants. Frequency of homocysteinemia for the $C677T$ mutation was 67/360 (18.6%). Homocysteinemia levels were elevated in homozygous compared with heterozygous subjects or those without the mutation ($20.6 \pm 18.8$ vs. $9.4 \pm 3.2$ μmol/l; $P < 0.0001$). The number of subjects with vitamin B12 deficiency ($<150$ pmol/l) was significantly higher among the homozygote than the heterozygote subjects or subjects without mutation (20.6%/27/293 vs. 29.3%/20/67; $P < 0.0001$). Homocysteinemia subjects had 4.2 times higher probability of having B12 deficiency (95% confidence interval = 2.1–8.3). Forearm endothelial function was assessed in 33 homozygote and 12 control subjects. Abnormal endothelial function was observed in homozogyous subjects and was worse in homocysteinemia subjects with vitamin B12 deficiency. Endothelial function was normalized after B12 and folic acid treatment. We found that homocysteinemia for the $C677T$ mutation is strongly associated with B12 deficiency. Coexistence of homozygosity for the $C677T$ mutation and B12 deficiency is associated with endothelial dysfunction and can be corrected with vitamin B12 and folic acid treatment.

homocysteinyg. endothelial dysfunction

THE RELEVANCE OF HOMOCYTEINE to atherothrombosis-related syndromes has long been debated. On one hand, an ample body of epidemiological evidence suggests a significant association between homocysteinemia and cardiovascular morbidity and mortality, although large-scale studies and several meta-analyses have failed to confirm a significant association (1, 7, 13, 22, 26, 43). Recent studies that examined the effect of homocysteine reduction on outcome events have also yielded conflicting results (4, 6, 28, 39). A possible explanation for differences in study outcomes is the definition of homocysteinemia and differences in the populations studied. Homocysteinuria syndrome, the most severe form of disturbed homocysteine metabolism, is a result of mutations in cystathionine β-synthase and is associated with high homocysteine and methionine levels and is strongly associated with early and often fatal development of atherothrombosis (23, 31, 45). Mutations of other genes that are involved in homocysteine metabolism and dietary intake of vitamin B12 and folic acid (6, 32) are associated with less severe homocysteinemia (42).

The early description of the $C677T$ mutation in the methylenetetrahydrofolate reductase (MTHFR) gene and the associated homocysteinemia aroused considerable interest. It was initially thought that the development of atherosclerosis in a significant number of patients could be explained on the basis of the mutation and associated homocysteinemia (19, 20). Recent studies have showed that the mutation and the homocysteinemia are moderately associated with stroke in some populations and are associated with endothelial cell dysfunction, but the relevance to coronary artery disease is still an open issue (1, 7).

In a study focused on homocysteine metabolism abnormalities and cardiac syndrome X (35), we observed that, in a control group of asymptomatic individuals, vitamin B12 deficiency was frequent in those who were homozygote for the MTHFR $C677T$ mutation. The present study aimed to test the hypothesis that homocysteinemia to the MTHFR $C677T$ mutation is associated with vitamin B12 deficiency and endothelial dysfunction. To test our hypothesis, we examined serum levels of homocysteine, vitamin B12, and folic acid and the presence of $C677T$ mutation in normal individuals with no symptoms attributed to coronary, brain, or peripheral artery disease. In a subgroup of individuals that were homozygous for the $C677T$ mutation with concomitant B12 deficiency, we studied forearm endothelial cell function before and after correcting B12 deficiency and after treatment with folic acid. Presence of concurrent abnormalities in homocysteine metabolism combined with endothelial dysfunction in a significant number of healthy individuals might provide insight into the etiology of high homocysteine levels and endothelial dysfunction in asymptomatic individuals.

METHODS

Study population. Two cohorts of patients comprised the study population. Volunteers were recruited if they had no history of chest pain, shortness of breath, stroke, or claudication. Individuals with
hypertension and diabetes were included in the study. Individuals treated with folic acid or vitamin B6 or B12 were excluded from the study.

The first cohort was composed of 155 asymptomatic volunteers recruited from the staff and volunteering organization of the participating medical center. Mean age was 54 ± 9 yr, and 66% were women. This cohort was originally recruited as controls for an earlier study conducted by our group (35). From observations made in this cohort, we recruited a second cohort of 205 asymptomatic volunteers from four industrial organizations. The mean age of volunteers in the second cohort was 37 ± 12 yr, and 66% were men. Total study population comprised 360 asymptomatic volunteers (age 44 ± 13 yr, 52% were men) (Table 1).

The study was reviewed and approved by the Lady Davis Carmel Medical Center Ethical Review Board (approval number 25796). All participants agreed to take part in the study by signing an informed consent.

Study protocol. Fasting blood samples were taken from all 360 volunteers. Genomic DNA was extracted from whole blood and was analyzed for the MTHFR C677T mutation. Serum levels of vitamin B12 and folic acid and plasma levels of homocysteine, glucose, cholesterol, high-density lipoprotein cholesterol, triglycerides, creatinine, and urea were measured. The frequency of the C677T mutation was determined in the study population, and the association between the mutation and homocysteine, folic acid, and vitamin B12 levels were examined.

After completion of laboratory testing, we studied endothelial cell function in 33 of 39 homozygote individuals in the second cohort. Six homozygous individuals were not included in this part of the study because they could not participate in the endothelial function studies because of time constraints. In the homozygote group, there were 12 individuals with vitamin B12 ≤ 150 pmol/l (group A) and 21 individuals with vitamin B12 > 150 pmol/l (group B).

Endothelial function was tested in 12 individuals that were either heterozygote to C677T mutation or had no mutation and had had vitamin B12 levels > 150 pmol/l (control group = group C).

Forearm endothelial function via high-resolution ultrasound was measured in the three groups at baseline. In group A, endothelial function and vitamin B12, homocysteine, and folic acid levels were measured two more times: after supplementation with vitamin B12 (reaching levels of B12 > 200 pmol/l) and after treatment with folic acid (5 mg daily for 12 wk). In group B, endothelial function was measured a second time after treatment with folic acid (5 mg daily for 12 wk). (Seven individuals in group B with vitamin B12 levels of 151–200 pmol/l were treated with vitamin B12 before treatment with folic acid.)

Blood sampling and genetic analysis. Twelve-hour fasting venous blood was collected in tubes containing disodium EDTA. Samples were promptly centrifuged (1,500 rpm for 10 min) after collection and stored at −20°C. In the first cohort, plasma homocysteine levels were measured as total homocysteine by amino acid analyzer (Biochrom 20, Pharmacia) (5, 25). In the second cohort, homocysteine was measured by fluorescence polarization immunoassay (Abbott, Chicago, IL) (10). We used radioimmunoassay (Dual Count SPNB, DPC) for blood analyses of vitamin B12 and folic acid levels. Glucose, cholesterol, triglycerides, creatinine, and urea levels were analyzed by standard methods (Hitachi 747, Boehringer Mannheim). Genomic DNA was extracted from venous blood cells with the use of the High Pure PCR preparation kit (Boehringer Mannheim). DNA was amplified by PCR using Taq DNA polymerase and suitable primers. For the mutation in the MTHFR gene, the amplified fragments were restricted with HinfI, which recognizes the C677T mutation as described by Frost et al. (14). After restriction, DNA fragments were separated by electrophoresis in 8% polyacrylamide gel.

Endothelial function studies. Endothelial function was assessed noninvasively as previously described by Sorensen et al. (40). High-resolution ultrasound was used to measure changes in arterial diameter in response to increased flow-mediated dilatation (FMD). We also measured nitrate-mediated dilatation using glyceryl trinitrate. Scans were obtained after 15 min of rest in the supine position, and subjects remained supine until the final scan was recorded. The brachial artery was scanned using longitudinal views by B-mode ultrasound imaging with the use of a 10-MHz linear array transducer. The center of the vessel was identified when the clearest images of the anterior and posterior walls of the artery were obtained, and the transmit zone was set to the level of the anterior wall. Depth and gain were optimized to identify the lumen-to-vessel wall interface and were kept constant until the final recording was obtained. Changes in diameter were assessed by M-mode from four consecutive cardiac cycles, and measurements from all four cycles were averaged. Scans were ob-

Table 1. Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>Baseline Data</th>
<th>All Study Population (n = 360)</th>
<th>Group A (n = 12)</th>
<th>Group B (n = 21)</th>
<th>Group C (n = 12)</th>
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<tbody>
<tr>
<td>Age, yr</td>
<td>44±14</td>
<td>33±12</td>
<td>40±16</td>
<td>40±13</td>
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<tr>
<td>Body-mass index</td>
<td>25.2±4.2</td>
<td>24.4±3.7</td>
<td>25.1±5.5</td>
<td>24.1±3.5</td>
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<tr>
<td>Female gender</td>
<td>47.8%</td>
<td>1/12</td>
<td>6/21</td>
<td>2/12</td>
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<tr>
<td>CVD risk factors, % of individuals</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Hypertension</td>
<td>17.8%</td>
<td>16.6%</td>
<td>7%</td>
<td>16.6%</td>
<td></td>
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<tr>
<td>Hyperlipidemia</td>
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<td>7%</td>
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<td>Diabetes mellitus</td>
<td>5%</td>
<td>0</td>
<td>4.7%</td>
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<tr>
<td>Smoking</td>
<td>29%</td>
<td>25%</td>
<td>42%</td>
<td>25%</td>
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<tr>
<td>Family history of CVD</td>
<td>30%</td>
<td>66%†</td>
<td>29%</td>
<td>17%†</td>
<td></td>
</tr>
<tr>
<td>Baseline biochemistry profile</td>
<td></td>
<td></td>
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<tr>
<td>Glucose, mg/dl</td>
<td>93±20</td>
<td>88±8</td>
<td>91±15</td>
<td>90±7</td>
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<tr>
<td>Creatinine, mg/dl</td>
<td>1.03±0.1</td>
<td>1.1±0.1</td>
<td>1.1±0.2</td>
<td>1.1±0.1</td>
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<tr>
<td>LDL cholesterol, mg/dl</td>
<td>128±35</td>
<td>105±17</td>
<td>134±32</td>
<td>116±20</td>
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<tr>
<td>Triglycerides, mg/dl</td>
<td>138±82</td>
<td>124±65</td>
<td>119±62</td>
<td>146±137</td>
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<tr>
<td>HDL cholesterol, mg/dl</td>
<td>51±13</td>
<td>44±13</td>
<td>57±11</td>
<td>55±18</td>
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<tr>
<td>Homocysteine metabolism</td>
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<td>Vitamin B12 levels, pmol/l</td>
<td>268±156</td>
<td>112±28*</td>
<td>219±60</td>
<td>453±261*</td>
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<tr>
<td>Folic acid, nmol/l</td>
<td>21±10</td>
<td>12±8</td>
<td>17±7</td>
<td>20±7</td>
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<tr>
<td>Homocysteine, μmol/l</td>
<td>11.5±9.6</td>
<td>39±24*</td>
<td>15±16</td>
<td>9.4±2.2*</td>
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<td>C677T mutation homozygosity</td>
<td>18.6%</td>
<td>100%</td>
<td>100%</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD. CVD, cardiovascular disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Group A, the homozygote group with vitamin B12 levels ≤150 pmol/l; group B, homozygote group with vitamin B12 levels >150 pmol/l; group C, control group. *P < 0.001 between groups A, B, and C. †P < 0.05 between groups A, B, and C.

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Dichotomous variables were compared by the \( t \)-test, and 14.7 \%/H11006\) MTHFR genotype, only history of coronary artery disease, gender, folic acid levels, and hypertension, low-density lipoprotein levels, age, family history of coronary artery disease, and gender. On the basis of the number of patients with vitamin B12 deficiency, we included in the logistic model, at the most, four independent variables.

**RESULTS**

Genotype, homocysteine, and B12 levels. Frequency of homozygosity for the \( C677T \) mutation (CC alleles) was 67/360 (18.6\%) in the study population. Frequency of heterozygosity was 162/360 (45\%), and frequency of subject with TT alleles was 131/360 (36.4\%).

Number of subjects with vitamin B12 deficiency (\( \geq150 \) pmol/l) was significantly higher among the homozygous group [20/67 (29.9\%)] than among heterozygous or subjects without the mutation [27/293 (9.2\%); \( P < 0.0001 \)]. Homocysteine levels were elevated in homozygote compared with heterozygote subjects or subjects without the mutation (20.6 \( \pm \) 18 vs. 9.4 \( \pm \) 3.2 \( \mu \)mol/l; \( P < 0.0001 \)). The number of subjects with homocysteinemia (\( >15 \) \( \mu \)mol/l) was significantly higher among the homozygote subjects than the heterozygote subjects or subjects without mutation [31/67 (46\%) vs. 11/293 (3.75\%); \( P < 0.0001 \)]. Frequency of homocysteinemia and the frequency of vitamin B12 deficiency in homozygote subjects were similar in the two cohorts studied, confirming the validity of our observation (8/28 in the first cohort and 14/39 in the second cohort).

In a logistic regression model that included diabetes mellitus, hypertension, low-density lipoprotein levels, age, family history of coronary artery disease, gender, folic acid levels, and MTHFR genotype, only \( C677T \) homozygosity [odds ratio (OR) 4.2 (95\% confidence limits: 2.1–8.3)], female gender [OR 0.43 (0.21–0.87)], and family history [OR 1.93 (0.99–1.93)] (borderline significance) were associated with vitamin B12 deficiency.

**Endothelial cell function.** FMD in group A (\( n = 12 \)) was significantly reduced vs. that shown for subjects in group B (\( n = 21 \)), and both were abnormal vs. that shown in individuals in group C (\( n = 12 \)) [3.3 \( \pm \) 1.0 for group A, 5.9 \( \pm \) 2.6\% for group B, and 14.7 \( \pm \) 2.9\% for group C].

![Fig. 1. Flow-mediated dilatation (FMD) in patients homozygous to the \( C677T \) mutation.](http://ajpheart.physiology.org/)

**DISCUSSION**

In this study, we demonstrated high prevalence of B12 deficiency in asymptomatic homozygote individuals for the MTHFR\(C677T\) mutation. We also demonstrated that individuals with concomitant B12 deficiency and homozygosity for the MTHFR\(C677T\) mutation had high levels of homocysteine and severe forearm flow-mediated endothelial dysfunction.

Population prevalence of vitamin B12 deficiency. The prevalence of B12 deficiency in an Israeli population was studied by Figlin et al. (11) and Gielchinsky et al. (16). The first group studied elderly individuals attending 19 geriatric day centers and 104 healthy younger controls. Based on cobalamin levels <147 pmol/l, the prevalence of vitamin B12 deficiency was 21\% (160/749) in the elderly population and 2.8\% (3/104) in normal controls. When the criteria for B12 deficiency was defined as cobalamin level <147 pmol/l and methylmalonic acid level >0.24 mmol/l, frequency in elderly visitors of the...
Vitamin B12 Deficiency Is Associated with MTHFR C677T Mutation

Mechanisms by which homocysteine interferes with endothelial function were studied by Stamler et al. (41). In their study, prolonged (>3 h) exposure of endothelial cells to homocysteine results in impaired endothelial-derived relaxing factor responses. In vitro, homocysteine increased smooth muscle cell proliferation and collagen production by these cells (30). Increased collagen deposition and smooth muscle proliferation can impair vascular reactivity and lead to homocysteine-related atherogenicity (30).

Heterozygote to disrupted MTHFR mice that were fed with a high-methionine, low-folate diet demonstrated altered tissue methylation capacity, higher propensity to homocysteinemia, and endothelial dysfunction (9). Reduced folate levels in individuals with C677T mutation were observed by Jacques et al. (18). Whether low levels were secondary to increased turnover or due to reduced dietary intake could not be fully concluded from their data. From the above mouse and human observations of dual abnormality of homocysteine metabolism, we can imply that our observation of high frequency of concomitant vitamin B12 deficiency in homozygotes may represent a small but important population of individuals with high homocysteine levels and endothelial cell dysfunction who are at a higher risk for vascular morbidity ascribed to homocysteinemia. The effect of homocysteine-lowering therapy as a method of primary prevention should be tested in such a selected group of individuals.

Loscalzo (29) suggested three possible mechanisms for failure of folic acid and vitamin B supplementation to reduce cardiovascular events in large-scale studies. The first mechanism is related to the property of folic acid to enhance cell proliferation as shown by its aggravation of in-stent restenosis (24). The second possible mechanism by which folic acid and vitamin B12 can accelerate atherosclerotic plaque development is highly relevant to our findings. Folic acid and vitamin B12 in the setting of mild homocysteinemia can change the rate of DNA methylation in vascular cells and lead to phenotypic changes in these cells and secondary plaque expansion. In the selected group of individuals that we identified with very high homocysteine levels, folic acid and vitamin B12 had a beneficial vascular effect. The third suggested mechanism is related to methylation of l-arginine and inhibition of nitric oxide synthase (29). Here again, in the group of individuals that we identified, vasodilation was improved dramatically with therapy.

The importance of combining vitamin B12 and folic acid treatment was demonstrated by Flicker et al. (12) and Quinlivan et al. (36). In their studies, they did not test for MTHFR mutations. On the basis of our findings, it is likely that they observed therapeutic effects in patients with dual abnormality in homocysteine metabolism. Our observation highlights the importance of identification of individuals that have dual abnormality of homocysteine metabolism by examining both MTHFR genotype and vitamin B12 levels. High homocysteine levels and disturbed endothelial function are characteristic of the population with dual abnormality. Unlike other works in which unselected populations were treated with folic acid and B12 and showed no benefit with this treatment, we suggest that therapy in the population that we identified can have significant benefits. Larger studies are needed to confirm our observation.

Endothelial-dependent vasodilation was severely disturbed in the homozygote individuals and was more pronounced with...
concomitant B12 deficiency. These patients had high frequency of moderate to severe homocysteinemia that can explain endothelial dysfunction. Correction of vitamin B12 deficiency improved endothelial function, but only treatment with folic acid fully normalized endothelial cell function in the individuals with dual abnormality in homocysteine metabolism (group A).

In our study, we identified a group of individuals homozygous for the MTHFR C677T mutation and have concomitant B12 deficiency. As a result of the dual abnormality in homocysteine metabolism, these individuals were found to have moderate and severe homocysteinemia. Whether vitamin B12 deficiency is a result of the abnormal homocysteine metabolism secondary to the MTHFR mutation or whether the concomitant abnormalities are related to some genetic association is difficult to determine based on our data.

Dual homocysteine metabolism abnormality in 5.6% of our group of asymptomatic individuals was associated with endothelial cell dysfunction. It may well be that these patients are the ones that develop cardiovascular syndromes as a consequence of prolonged moderate and severe homocysteinemia. These patients responded dramatically to vitamin B12 and folic acid treatment and demonstrated normalization of endothelial cell function with treatment. The step-wise improvement of endothelial function indicated that both vitamin B12 and folic acid are necessary for normalization of endothelial function.

Although the association of endothelial cell dysfunction and the development of atherosclerosis are not fully defined, it is clear that chronic endothelial cell dysfunction is a marker of vascular pathology that is also seen with classical risk factors for atherosclerosis such as hypertension and hyperlipidemia (8, 34). Failure of large-scale studies, in unselected populations, to demonstrate reduced cardiovascular risk and events with homocysteine-lowering therapy may be because only patients with dual-homocysteine abnormality can potentially benefit from this therapy. We showed that dual abnormality could be found in 5.6% of asymptomatic individuals. It is reasonable to suggest that vitamin B12 and folic acid should be targeted to these individuals and that outcome of such therapy in an unselected population may mask the beneficial effects in the specific group of individuals with dual-homocysteine metabolism abnormalities.

In summary, individuals with dual abnormality of homocysteine metabolism were found to have moderate and severe homocysteinemia. Treatment with vitamin B12 and folic acid resulted in significant reduction in homocysteine and normalization of endothelial function. Future studies of the effects of folic and vitamin B12 therapy will provide information on the role of this treatment in large population; however, we suggest that, in a subgroup of individuals with dual abnormality in homocysteine metabolism, folic acid and vitamin B12 therapy are effective in normalization of homocysteine levels and endothelial cell function.

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GRANTS

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