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 homocysteine for the methylenetetrahydrofolate reductase (MTHFR) C677T mutation and vitamin B12 deficiency. Forearm endothelial function and homocysteine were measured in study participants. Frequency of homocysteinemia for the C677T mutation was 67/360 (18.6%). Homocysteine levels were elevated in homozygous compared with heterozygous subjects or those without the mutation (20.6 ± 18.8 vs. 9.4 ± 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 [20/67 (29.8%) vs. 27/293 (9.2%); P < 0.0001]. Homocysteine 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 homozygous subjects and was worse in homozygote 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 homocysteinemia for the C677T mutation and B12 deficiency is associated with endothelial dysfunction and can be corrected with vitamin B12 and folic acid treatment.

homocysteine; endothelial function

The relevance of homocysteine 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...
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hypothesis 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 and homocysteine, folic acid, and vitamin B12 levels was determined in the study population, and the association between the mutation and homocysteine, folic acid, and vitamin B12 levels was 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 Hinfl, which recognizes the C677T mutation as described by Frostell 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-
Dichotomous variables were compared by the paired t-test. Paired variables were compared between groups using Wilcoxon rank sum test. Interobserver and intraobserver variabilities were reported in previous work (2, 3).

**Statistical analysis.** Results are shown as means ± SD. Continuous variables were compared between groups using Wilcoxon rank sum test. Paired t-test was used for analysis of data before and after treatment in which each individual served as his or her own control. Dichotomous variables were compared by the x² test. For comparison between groups A, B, and C, the all-pair-wise Tukey’s method was used (Statistix 8 software). Logistic regression analysis was used to test the association between vitamin B12 deficiency and homozgyosity for the C677T mutation. In the logistic model, we also tested the association of vitamin B12 deficiency with diabetes mellitus, hypertension, low-density lipoprotein levels, folic acid, 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 homozgyosity 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 (≤150 pmol/l) was significantly higher among the homozygous group [20/67 (29.8%)] 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 ± 18 vs. 9.4 ± 3.2 μmol/l; P < 0.0001). The number of subjects with homocysteinemia (>15 μmol/l) was significantly higher among the homozgyote subjects than the heterozygote subjects or subjects without mutation [31/67 (46%) vs. 11/293 (3.75%); P < 0.0001]. Frequency of homozygous individuals 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 homozgyosity [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 ± 1.0 for group A, 5.9 ± 2.6% for group B, and 14.7 ± 2.9% for group C; 1-way analysis of variance (AOV), P < 0.00001, Tukey’s honestly significant different all-pair-wise comparisons test, α 0.05]. No significant difference was found among groups A, B, and C in nitrate-mediated dilatation (16.5 ± 7.7%, 18.8 ± 7.6%, 22.3 ± 3.9%; P = not significant).

**Response to vitamin B12 supplementation in group A.** Individuals in group A were treated with vitamin B12, 1 mg sublingual tablets, until B12 levels reached >200 pmol/l. Homocysteine levels were significantly reduced after B12 supplementation (39.1 ± 23.5 vs. 21.8 ± 1.9 μmol/l; paired t-test, P < 0.0001). FMD was significantly improved (3.3 ± 1.0 vs. 8.1 ± 1.5%; paired t-test, P < 0.0001) (Fig. 1).

**Response to folic acid treatment in groups A and B.** Treatment with 5 mg of folic acid daily for 12 wk in individuals in groups A and B resulted in a significant increase in folic acid levels (15.0 ± 7.6 vs. 34.6 ± 8.1 nmol/l; paired t-test, P < 0.0001) and significant reduction in homocysteine levels (24.1 ± 22.4 vs. 8.8 ± 4.1 μmol/l; paired t-test, P < 0.002). Further significant increase in FMD in group A (8.1 ± 1.5 vs. 13.6 ± 3.4%; paired t-test, P < 0.0001) (Fig. 1) and a significant increase in group B (5.8 ± 2.6 vs. 12.9 ± 3.4%; paired t-test, P < 0.0001) were observed with folic acid treatment. After completion of vitamin B12 and folic acid therapy, FMD was similar in groups A, B, and C (baseline) (13.6 ± 3.4, 12.8 ± 3.7, 14.7 ± 2.3; P = not significant).

**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 homozgyosity 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...
geriatric day care was 12.6% and frequency of younger control was 1.92% (11). The relatively high frequency of vitamin B12 deficiency in elderly patients visiting day care clinics is in sharp contrast to normal controls in this study. Our study population is probably similar to the healthy younger control population and not to the elderly population; therefore, low frequency of B12 deficiency can be expected.

Gielchinsky et al. (16) used 200 pmol as the cutoff level for B12 deficiency and studied several groups of Israelis with emphasis on ethnic origins. The reported prevalence varied from 12% in individuals from Moroccan origin to 37.5% in individuals from Tunisian origin. The prevalence in 270 individuals from Ashkenazi origin was 22.3%. The differences in prevalence of B12 deficiency between our work (10%) and the work of Gielchinsky et al. can be explained on the basis of the different cutoff points defined for deficiency. Goland et al. (17) reported 30% vitamin B12 deficiency in patients undergoing coronary angiography, but they also used 200 pmol/l as the cutoff level for defining vitamin B12 deficiency (17). Average vitamin B12 levels in our two cohorts studied were higher than those reported by Kark et al. (21) in individuals from Jerusalem. 

Vitamin B12 levels in homozygote individuals for the C677T mutation. The main finding of our study is the high prevalence of B12 deficiency in C677T homozygotes. The similarly high rate (30%) of homozygosity with concomitant B12 deficiency in the two cohorts lends support to the validity of our observation. In our study, moderate and severe homocysteinemia were more frequent in homozygote individuals and even more so in homozygote individuals with concomitant vitamin B12 deficiency. Frequency of homozygosity in the two cohorts was similar, but B12 deficiency tended to be higher in the second larger cohort, which was mostly pronounced in men. The demographic and health profile differences between the two cohorts reflect the different recruitment sites, with a majority of younger male volunteers recruited for the second cohort from the industrial organizations. The higher prevalence of vitamin B12 deficiency in men was also observed by Figlin et al. (11) in an Israeli population and by Ganji and Kafai (15) in the NHANES III survey.

The correlation between serum homocysteine levels and cardiovascular syndromes is controversial (7, 13, 22, 33, 38). Although initial therapeutic trials directed at reduction of homocysteine levels showed reduction in cardiovascular events, further studies failed to show beneficial effects and demonstrated worsening of outcome in patients after angioplasty or myocardial infarction treated with folic acid and vitamin B (4, 24, 27, 39). The effect of folic acid and vitamin B6 and 12 supplementation on risk reduction of stroke was considered by the Heart Outcomes Prevention Evaluation (HOPE)-2 investigators to possibly be spurious, but their findings of borderline stroke risk reduction are in agreement with meta-analyses of studies of homocysteine and risk of stroke (1, 37, 43). Additional questions regarding the dosage of therapy and interactions among vitamins B6 and 12 and folic acid used in the HOPE-2 and Norwegian Vitamin (NORVIT) trials were raised by Wang et al. (44), who concluded that vitamin B and folic acid therapies should be targeted to patients with higher than average homocysteine levels and not to the general population of cardiovascular patients.

Mechanisms by which homocysteine interferes with endothelial function were studied by Stamlser 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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REFERENCES

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