Association of Vitamin B12 Deficiency with Homozygosity of the TT *MTHFR* C677T Genotype, Hyperhomocysteinemia, and Endothelial Cell Dysfunction

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ABSTRACT: **Background:** Hyperhomocysteinemia is associated with increased cardiovascular risk, but treatment with folic acid has no effect on outcome in unselected patient populations.

Objectives: To confirm previous observations on the association of homozygosity for the TT *MTHFR* genotype with B12 deficiency and endothelial dysfunction, and to investigate whether patients with B12 deficiency should be tested for 677MTHFR genotype.

Methods: We enrolled 100 individuals with B12 deficiency, tested them for the *MTHFR* C677T polymorphism and measured their homocysteine levels. Forearm endothelial function was checked in 23 B12-deficient individuals (13 with TT *MTHFR* genotype and 10 with CT or CC genotypes). Flow-mediated dilatation (FMD) was tested after short-term treatment with B12 and folic acid in 12 TT *MTHFR* homozygotes.

Results: Frequency of the TT *MTHFR* genotype was 28/100 (28%), compared with 47/313 (15%) in a previously published cohort of individuals with normal B12 levels ($P = 0.005$). Mean homocysteine level was 21.2 ± 16 μM among TT homozygotes as compared to 12.3 ± 5.6 μM in individuals with the CC or CT genotype ($P = 0.008$). FMD was abnormal (≤ 6%) in 9/13 TT individuals with B12 deficiency (69%), and was still abnormal in 7/12 of those tested 6 weeks after B12 and folic treatment (58%).

Conclusions: Among individuals with B12 deficiency, the frequency of the TT *MTHFR* genotype was particularly high. The TT polymorphism was associated with endothelial dysfunction even after 6 weeks of treatment with B12 and folic acid. Based on our findings we suggest that B12 deficiency be tested for MTHFR p*olymor*phism in order to identify potential vascular abnormalities and increased cardiovascular risk.

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KEY WORDS: homocysteine, B12 deficiency, endothelial function, *MTHFR* polymorphism, cardiovascular risk

Epidemiologic studies have suggested that hyperhomocysteinemia is associated with increased risk for cardiovascular disease, independent of classic cardiovascular risk factors [1]. High levels of homocysteine have been associated with oxidative stress, endothelial damage and enhanced thrombogenicity [2], yet randomized clinical trials did not show any benefit of homocysteine-lowering therapy on cardiovascular events and outcome [3,4]. Vascular disease, and not hyperhomocysteinemia, was the main inclusion criterion for these randomized trials.

C-to-T substitution at base 677 of the 5,10-methylene tetrahydrofolate reductase (*MTHFR*) gene has been associated with mild hyperhomocysteinemia (15–25 μmol/L) and with coronary artery disease [5]. However, a meta-analysis of 13 studies demonstrated the TT *MTHFR* genotype to be associated with mild hyperhomocysteinemia but not with increased cardiovascular risk [6]. Mager [7] criticized this meta-analysis for pooling data without considering such factors as ethnicity and geography or specific outcomes. In their own study, conducted in Israel, Mager et al. [8] found that TT homozygosity for *MTHFR* was associated with an increased risk of premature coronary artery disease but not with overall disease risk. A meta-analysis concluded that reported associations of TT *MTHFR* C677T polymorphism with coronary heart disease may be due to a publication bias [9]. Nevertheless, associations between MTHFR genotypes and cardiovascular risk may exist for specific outcomes, with relevance for certain populations.

In our earlier study of asymptomatic individuals [10] we found a significantly higher rate of B12 deficiency, as well as a higher mean homocysteine level among those with the TT *MTHFR* genotype than those without this genotype. Endothelial function, demonstrated by flow-mediated dilatation (FMD), was impaired among TT homozygotes with B12 deficiency compared to those without. The current prospective study aimed to confirm the association between B12 deficiency and the 677*MTHFR* genotype and to investigate whether patients

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with B12 deficiency should be tested for 677*MTHFR* genotype. Identification of patients with dual homocysteine metabolism abnormalities and moderate hyperhomocysteinemia is important because these patients, as shown in the present study, have abnormal endothelial cell function and may benefit from long-term supplementation of B12 and folic acid.

PATIENTS AND METHODS

To test our hypothesis we enrolled 101 consecutive individuals with B12 deficiency (B12 < 150 pmol/L). All patients were tested for B12 levels by their primary physician. We identified the patients using the database of the northern Israel regional laboratory of Clalit Health Services, the largest health fund in Israel. Exclusion criteria were age below 20 or above 60 years, angina pectoris or known coronary artery disease, diabetes mellitus, and familial hypercholesterolemia. One individual was excluded from the study after enrollment when it was revealed that he had previously undergone percutaneous coronary intervention. The final study group comprised 100 individuals. The study was approved by the institutional review board and all participants signed informed consent (http://clinicaltrials.gov/ct2/show/NCT00730574).

STUDY PROTOCOL

Fasting blood samples were taken from all 100 individuals. Genomic DNA was extracted from whole blood and analyzed for the *MTHFR* C677T polymorphism with taqman SNP genotyping assay by design, manufactured by Applied Biosystems, USA (ABI). Analysis was performed on a 7500 fast real-time polymerase chain reaction instrument (ABI). Serum levels of vitamin B12, folic acid, glucose, cholesterol, high density lipoprotein-cholesterol, triglycerides and plasma levels of homocysteine were measured [9]. Endothelial cell function and exercise testing were performed in 10 individuals with CC or CT *MTHFR* genotypes and in 13 with the TT genotype who had not yet started B12 therapy and who agreed to participate (the individuals who participated in the endothelial function part of the study were those who committed to attend the multiple visits that were required). All tests were repeated in 12 TT homozygotes after treatment with 1 mg daily of sublingual vitamin B12 and 5 mg daily of folic acid for 6 weeks (one homozygous individual did not arrive for re-testing).

ENDOTHELIAL FUNCTION STUDIES

Endothelial function was examined non-invasively in the brachial artery using high resolution ultrasound (Sonos 5500, Philips, Andover, MA, USA) [11]. We measured FMD, the change in brachial artery diameter in the hyperemic state, as a measure of endothelial function. The brachial artery was scanned longitudinally using a 3–10 MHz linear array transducer. Depth and gain settings were optimized and arterial diameter, media to media, was measured at end-diastole according to the QRS complex on the electrocardiogram. At least four cardiac cycles were averaged for each data point. Scans were obtained after a 10 minute rest (baseline), during reactive hyperemia, after a 10 minute recovery, and 5 minutes after administration of 400 µg glyceryl trinitrate sublingual spray. Reactive hyperemia was induced by 5 minute inflation of a sphygmomanometer cuff on the forearm to a pressure of 300 mmHg. Vessel diameter was recorded 1 minute after cuff deflation. FMD was calculated as FMD (%)=[(D2-D1)/D1]x100, where D2 is the reactive hyperemia diameter and D1 the baseline diameter. Endothelial-independent dilatation (nitrate-mediated dilation, NMD) was calculated as NMD (%)=[(D3-D1)/D1]x100, where D3 is the diameter after nitrate administration. Scans were recorded digitally on a magneto-optic disk for off-line analysis. Measurements were performed by a highly qualified technician who was blinded to the study design, genetic status or treatment. Intra- and inter-observer variability for FMD measurements in our laboratory was reported previously and FMD ≤ 6% was considered abnormal [11].

EXERCISE TESTING

Symptom-limited treadmill exercise testing was performed using the Bruce protocol [12]. Exercise capacity (test duration in minutes), maximal heart rate, percent of maximal predicted heart rate, symptoms (angina pectoris or dyspnea) and maximal ST deviation were recorded.

STATISTICAL ANALYSIS

Continuous variables are presented as means ± SD and compared using the paired or unpaired *t*-test as appropriate. Categorical variables were compared using the Yate's corrected chi-square test. The frequency of the TT *MTHFR* C677T genotype in the study group was compared to that in asymptomatic individuals without B12 deficiency in a cohort with similar demographics, as reported by us previously [10]. Differences were considered statistically significant at the two-sided $P < 0.05$ level.

RESULTS

MTHFR C677T GENOTYPE DISTRIBUTION AND HOMOCYSTEINE LEVEL

In this cohort of B12-deficient individuals, the frequency of the TT *MTHFR* genotype polymorphism was 28/100 (28%), CT 48/100 (48%), and CC 24/100 (24%). This compares with a frequency of 47/313 (15%) for the TT genotype among individuals without vitamin B12 deficiency in an asymptomatic population that we studied previously [10] ($P = 0.005$). Characteristics of the study group are summarized in Table 1. The group with the *TT MTHFR* genotype had a significantly higher mean level of homocysteine than did the group with CT and CC combined (21.2 ± 16 vs. 12.3 ± 5.6 µM, $P = 0.008$).

Table 1. Baseline characteristics of individuals with B12 deficiency according to *MTHFR* C677T genotype

	Total group (n=100)	TT MTHFR (n=28)	CC and CT MTHFR (n=72)	*P* value
Age (yr)	41.3 ± 11.8	42.0 ± 12.1	40.8 ± 11.8	0.6
Males	48 (48%)	17 (61%)	31 (43%)	0.2
Homocysteine metabolism				
Homocysteine (µmol/L)	14.9 ± 10.5	21.2 ± 16	12.3 ± 5.6	0.008
B12 (pmol/L)	132 ± 16	140 ± 14	130 ± 16	0.07
Folic acid (nmol/L)	18.1 ± 10.7	15.6 ± 10.5	19.1 ± 10.7	0.15
New diagnosis of B12 deficiency	57 (57%)	14 (50%)	43 (60%)	0.5
CVD risk factors				
Smoking	28 (28%)	8 (29%)	20 (28%)	1.0
Hypertension	4 (4%)	1 (3.6%)	3 (4.2%)	1.0
Hyperlipidemia	7 (7%)	4 (14%)	3 (4.2%)	0.18
Family history of CVD	8 (8%)	2 (7.4%)	6 (8.7%)	1.0
Baseline biochemistry profile				
LDL cholesterol (mg/dl)	121 ± 34	124 ± 31	119 ± 35	0.56
HDL cholesterol (mg/dl)	53 ± 12	52 ± 12	53.2 ± 12.2	0.8
Triglycerides (mg/dl)	124 ± 69	124 ± 66	123 ± 71	0.96
Hemoglobin (g/dl)	13.9 ± 1.3	14.3 ± 0.94	13.7 ± 1.4	0.04
Glucose (mg/dl)	86.9 ± 10.1	87.1 ± 10.9	86.8 ± 9.8	0.9
Medical therapy				
Statins	4 (4%)	0	4 (5.6%)	
ACE–I/ARB	1 (1%)	1 (3.6%)	0	0.5
Calcium blocker	0	0	0	0.6
Aspirin	2 (2%)	1 (3.6%)	1 (1.4%)	1.0

ACE-I = angiotensin-converting enzyme inhibitor, ARB = angiotensin receptor blocker, CVD = cardiovascular disease

Table 2. Homocysteine metabolism, endothelial function, and exercise capacity according to MTHFR genotype, at baseline and after B12 therapy

	CT and CC *MTHFR* (n=10)	TT *MTHFR* at baseline (n=13)	TT *MTHFR* after treatment (n=12)
Age (yr)	43.0 ± 12.9	42.4 ± 12.5	41.3 ± 12.4
Males	5 (50%)	8 (62%)	8 (67%)
B12 (pmol/L)	135 ± 11	138 ± 15	505 ± 165
Folic acid (nmol/L)	12.5 ± 5.5	11.8 ± 4.7	≥ 54.4
Homocysteine (µmol/L)	15.5 ± 8.9	23.1 ± 12.9	9.1 ± 2.8
FMD (%)	5.2 ± 5.7	5.8 ± 3.1	6.4 ± 3.5
No. with normal FMD (> 6%)	4 (40%)	4 (31%)	5 (42%)
NMD (%)	15.8 ± 5.2	13.7 ± 4.2	13.0 ± 4.2
Exercise capacity, Bruce protocol	(n=10)	(n=13)	(n=10)
Exercise duration (min)	9.88 ± 2.2	9.40 ± 2.6	9.44 ± 2.7
Maximal heart rate (beats/min)	162 ± 14	166 ± 24	162 ± 24
% of maximal predicted heart rate	92.1 ± 7.2	92.9 ± 9.2	91 ± 8.3
Peak exercise systolic blood pressure (mmHg)	158 ± 29	170 ± 22	169 ± 21

FMD = flow-mediated dilatation, NMD = nitrate-mediated dilatation

ENDOTHELIAL FUNCTION AND RESPONSE TO VITAMIN B12 THERAPY

Characteristics of the 23 individuals (13 TT *MTHFR* and 10 CT or CC *MTHFR*) for whom response to B12 and folate therapy was investigated prospectively are presented in Table 2. One individual refused follow-up FMD and NMD testing. Mean serum levels of vitamin B12 and folic acid did not differ significantly between the groups at baseline, yet increased significantly for the TT *MTHFR* group following B12 and folate therapy. The mean plasma homocysteine level for the TT group at baseline was higher than for the CT/CC group ($P = 0.008$) and decreased after treatment ($P = 0.005$). No statistically significant differences were observed in mean FMD and NMD at baseline between those with TT and CT/CC genotype, nor before or after B12 therapy for the TT group.

EXERCISE CAPACITY AND VITAMIN B12 THERAPY

No statistically significant differences were observed in any of the mean levels of exercise capacity parameters tested by the Bruce protocol (exercise duration, maximal heart rate, percent of maximal predicted heart rate, peak exercise systolic blood pressure) between those with TT (n=13) and those with CC/CT *MTHFR* genotype (n=10), and before and after 6 weeks of B12 therapy for those with TT homozygosity (n=10, as two participants failed to show for the second test) [Table 2].

DISCUSSION

In this prospective study of 100 consecutive individuals with B12 deficiency we confirmed the association between B12 deficiency and the C677T *MTHFR* mutation. Our previous demonstration that *MTHFR* TT genotype is associated with B12 deficiency based on higher than expected frequencies in an asymptomatic population [10] does not imply that the TT genotype frequency will be higher in patients with B12 deficiency. In the current study we showed that 28% of B12-deficient patients have homozygosity for the TT *MTHFR* genotype. Among individuals with B12 deficiency, TT homozygosity was associated with a higher level of plasma homocysteine than among those with the CC or CT genotype. Our findings justify testing for the TT *MTHFR* genotype in individuals with B12 deficiency, since these individuals may have a 28% chance of concomitant B12 deficiency and TT genotype, a higher frequency of hyperhomocysteinemia and vascular dysfunction, and they may benefit from B12 and folic acid treatment and long-term cardiovascular focused follow-up.

The second important finding of our study was the high frequency of abnormal endothelial function in individuals with B12 deficiency, and the observation that abnormal endothelial function did not improve in individuals with TT *C677T* genotype and B12 deficiency, following 6 weeks of B12 and folic acid supplementation and reduction in homocysteine levels. A number of mechanisms have been suggested to explain the concurrence of endothelial dysfunction with abnormal vasodilation and enhanced thrombosis in hyperho-

mocysteinemia, such as reactive oxygen species and reduced proliferation of endothelial progenitor cells [13].

Our study cohort comprised individuals with B12 deficiency (< 150 pmol/L) who had been referred by their primary physician for B12 testing, generally because of non-specific complaints, such as fatigue. The frequency of B12 deficiency in our previous study population was 13% in healthy individuals who did not carry the T *MTHFR* allele [10]. Figlin et al. [14] reported B12 deficiency (< 147 pmol/L) in 2.8% of healthy individuals and 21% in an elderly population in Israel. It was found that C-to-T substitution at base 677 of *MTHFR* results in thermolability and reduction of enzyme activity [15], increased levels of plasma homocysteine [13] and increased susceptibility to hyperhomocysteinemia [14]. The underlying cause for the association between B12 deficiency and TT homozygosity is unknown. Possible explanations are increased B12 consumption secondary to abnormal homocysteine metabolism due to the T allele of *MTHFR* C677T polymorphism; and decreased intestinal absorption genetically associated with the C677T polymorphism. Further evidence for the association of homocysteine blood levels, *MTHFR* and the vitamin B group can be found in population-based genomic association tests [16]. The number of patients with abnormal endothelial function was higher among homozygotes; the difference was not statistically significant perhaps due to the relatively small sample size. In the current study we focused on *MTHFR* TT homozygotes, because their homocysteine levels were significantly higher and the risk of vascular abnormalities in patients with high homocysteine levels is more pronounced.

Folic acid treatment has been shown to reduce homocysteine levels [17] and was reported (with or without B12) to reduce mortality in patients with coronary artery disease and hyperhomocysteinemia in non-randomized trials [18]. A randomized controlled trial found folic acid, vitamin B12 and pyridoxine to significantly reduce homocysteine levels and to decrease the rate of restenosis and the need for revascularization of the target lesion after coronary angioplasty [19]. Large randomized trials of unselected patients who did not necessarily have the C677T polymorphism, hyperhomocysteinemia or B12 deficiency did not show any benefit of B12 and folic acid therapy in preventing cardiovascular events [4]. A recent meta-analysis confirmed the lack of effect of B12 supplementation on cardiovascular events [3]. Folic acid and B12 treatment can be harmful and can induce cell proliferation, secondary atherosclerotic plaque expansion and nitric oxide synthase inhibition [20]. Furthermore, non-selective treatment with folic acid and B12 was associated with cancer and increased mortality [21]. This downside of folic acid and B12 treatment underlines the importance of targeting its administration. The frequency of combined B12 deficiency and homozygosity for C677T polymorphism in our previous study population was 5.6% [10]. These individuals with dual abnormality of homocysteine metabolism together with moderate hyperhomocysteinemia and endothelial cell dysfunction could be a target for long-term homocysteine-reducing therapy.

The distinctiveness of the current study is its focus on B12-deficient individuals. Endothelial function was not shown to differ between TT *MTHFR* homozygotes and individuals carrying the C allele, nor did it improve significantly after 6 weeks of folic acid and B12 supplementation in B12-deficient individuals with TT *MTHFR* genotype. This contrasts with the findings of our previous study which showed lower baseline endothelial function among TT homozygotes who were B12 deficient than among TT homozygotes with B12 levels within the normal range. In that study, endothelial function was found to improve in TT homozygotes following 12 weeks of supplementation of B12 and folic acid [10]. Considering both studies, it seems that B12 deficiency had a greater impact on endothelial function than did the *MTHFR* genotype, and that 6 weeks of B12 and folic acid treatment is sufficient to reduce homocysteine levels yet insufficient to normalize endothelial function. This is reminiscent of other studies, albeit among individuals without B12 deficiency, in which B12 and folic acid treatment reduced homocysteine levels but did not affect cardiovascular risk [4].

For the TT *MTHFR* homozygotes with B12 deficiency in the present cohort, mean homocysteine levels were lower and folic acid levels higher than in our previously published cohort, 21.2 ± 16 vs. 39 ± 24 μmol/L and 15.6 ± 10.5 vs. 12 ± 8 nmol/L, respectively. These differences may result from longer duration of B12 deficiency and hyperhomocysteinemia described in the previous study. Men comprised 92% of the individuals with TT *MTHFR* homozygosity and B12 deficiency in that study, compared to only 61% in the present study. A higher prevalence of B12 deficiency in men was previously reported in cohorts both in Israel and the United States [14]. Lower mean levels of plasma homocysteine and higher levels of serum folate for women compared to men have been reported, e.g., in the Framingham Offspring cohort [22] and in a cohort of older adults in Israel [23].

Peripheral endothelial function, as measured by brachial artery reactivity, has been shown to be a marker of cardiovascular disease and a predictor of poor outcome from cardiovascular events [24]. On the other hand, Frick and co-authors [25] found no correlation between peripheral endothelial function and the extent of coronary disease on coronary angiography. Based on our findings, a large randomized trial, powered to detect treatment effect on overall survival in this selected group of subjects with dual abnormality in homocysteine metabolism and hyperhomocysteinemia, is needed to confirm the benefits of lowering homocysteine.

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References

1. Eikelboom JW, Lonn E, Genest J Jr, Hankey G, Yusuf S. Homocyst(e)ine and cardiovascular disease: a critical review of the epidemiologic evidence. *Ann Intern Med* 1999; 131: 363-75.
2. Welch GN, Loscalzo J. Homocysteine and atherothrombosis. *N Engl J Med* 1998; 338: 1042-50.
3. Galan P, Kesse-Guyot E, Czernichow S, Briancon S, Blacher J, Hercberg S. Effects of b vitamins and omega 3 fatty acids on cardiovascular diseases: a randomised placebo controlled trial. *BMJ* 2010; 341: c6273.
4. Bonaa KH, Njolstad I, Ueland PM, et al. Homocysteine lowering and cardiovascular events after acute myocardial infarction. *N Engl J Med* 2006; 354: 1578-88.
5. Frosst P, Blom HJ, Milos R, et al. A candidate genetic risk factor for vascular disease: a common polymorphism in methylenetetrahydrofolate reductase. *Nat Genet* 1995; 10: 111-13.
6. Brattström L, Wilcken DE, Ohrvik J, Brudin L. Common methylenetetrahydrofolate reductase gene polymorphism leads to hyperhomocysteinemia but not to vascular disease: the result of a meta-analysis. *Circulation* 1998; 98: 2520-6.
7. Mager A. Methylenetetrahydrofolate reductase gene and coronary artery disease. *Circulation* 2000; 101: E172-3. Comment on: Common methylenetetrahydrofolate reductase gene polymorphism leads to hyperhomocysteinemia but not to vascular disease: the result of a meta-analysis.
8. Mager A, Lalezari S, Shohat T, et al. Methylenetetrahydrofolate reductase genotypes and early-onset coronary artery disease. *Circulation* 1999; 100: 2406-10.
9. Mamasoula C, Prentice RR, Pierscionek T, et al. Association between C677T polymorphism of methylene tetrahydrofolate reductase and congenital heart disease: meta-analysis of 7697 cases and 13,125 controls. *Circ Cardiovasc Genet* 2013; 6: 347-53.
10. Zittan E, Preis M, Asmir I, et al. High frequency of vitamin b12 deficiency in asymptomatic individuals homozygous to *MTHFR* c677t polymorphism is associated with endothelial dysfunction and homocysteinemia. *Am J Physiol Heart Circ Physiol* 2007; 293: H860-5.
11. Amir O, Alroy S, Schliamser JE, et al. Brachial artery endothelial function in residents and fellows working night shifts. *Am J Cardiol* 2004; 93: 947-9.
12. Bruce RA, Hornsten TR. Exercise stress testing in evaluation of patients with ischemic heart disease. *Prog Cardiovasc Dis* 1969; 11: 371-90.
13. Cheng Z, Jiang X, Kruger WD, et al. Hyperhomocysteinemia impairs endothelium-derived hyperpolarizing factor-mediated vasorelaxation in transgenic cystathionine beta synthase-deficient mice. *Blood* 2011; 118: 1998-2006.
14. Figlin E, Chetrit A, Shahar A, et al. High prevalences of vitamin b12 and folic acid deficiency in elderly subjects in Israel. *Br J Haematol* 2003; 123: 696-701.
15. Rozen R. Genetic predisposition to hyperhomocystinemia: deficiency of methylenetetrahydrofolate reductase *(MTHFR)*. *J Thromb Haemost* 1997; 78: 523-6.
16. Tanaka T, Scheet P, Giusti B, et al. Genome-wide association study of vitamin b6, vitamin b12, folate, and homocysteine blood concentrations. *Am J Hum Genet* 2009; 84: 477-82.
17. Mayer O, Filipovsky J, Hromadka M, et al. Treatment of hyperhomocysteinemia with folic acid: effects on homocysteine levels, coagulation status, and oxidative stress markers. *J Cardiovasc Pharmacol* 2002; 39: 851-7.
18. Mager A, Orvin K, Koren-Morag N, et al. Impact of homocysteine-lowering vitamin therapy on long-term outcome of patients with coronary artery disease. *Am J Cardiol* 2009; 104: 745-9.
19. Schnyder G, Roffi M, Pin R, et al. Decreased rate of coronary restenosis after lowering of plasma homocysteine levels. *N Engl J Med* 2001; 345: 1593-600.
20. Loscalzo J. Homocysteine trials – clear outcomes for complex reasons. *N Engl J Med* 2006; 354: 1629-32.
21. Ebbing M, Bonaa KH, Nygard O, et al. Cancer incidence and mortality after treatment with folic acid and vitamin b12. *JAMA* 2009; 302: 2119-126.
22. Jacques PF, Bostom AG, Wilson PW, Rich S, Rosenberg IH, Selhub J. Determinants of plasma total homocysteine concentration in the Framingham Offspring cohort. *Am J Clin Nutr* 2001; 73: 613-21.
23. Dankner R, Chetrit A, Dror GK, Sela BA. Physical activity is inversely associated with total homocysteine levels, independent of C677T *MTHFR* genotype and plasma B vitamins. *Age* 2007; 29: 219-27.
24. Brevetti G, Silvestro A, Schiano V, Chiariello M. Endothelial dysfunction and cardiovascular risk prediction in peripheral arterial disease: additive value of flow-mediated dilation to ankle-brachial pressure index. *Circulation* 2003; 108: 2093-8.
25. Frick M, Schwarzacher SP, Alber HF, et al. Morphologic rather than functional or mechanical sonographic parameters of the brachial artery are related to angiographically evident coronary atherosclerosis. *J Am Coll Cardiol* 2002; 40: 1825-30.