

Methylenetetrahydrofolate Reductase Enzyme Mutations and Relationship of Homocysteine Vitamin B₁₂ and Folate Blood Levels

C. Tuba GÜNEBAK ŞAHİN¹, Berkan SAYAL², Erdal COŞGUN³, H. Tanju BESLER¹, M. Sinan BEKSAÇ²

Ankara, Turkey

OBJECTIVE: The purpose of this study is to investigate the influence of MTHFR gene polymorphisms which may effect the interaction in between homocysteine, folic acid and vitamin B₁₂ metabolisms.

STUDY DESIGN: The subject of this study is consisted of 165 patients who were obtained from "Hacettepe University Maternal Fetal Medicine Data Base". These were married women of childbearing age with MTHFR gene polymorphisms who experienced bad obstetrical history. Plasma total homocysteine, serum folic acid and vitamin B₁₂ levels of the subjects were analysed and the interrelation between these nutritional factors was examined.

RESULTS: Both folic acid and vitamin B₁₂ are significantly important and effective factors in homocysteine metabolism in MTHFR mutant subjects.

CONCLUSION: Folic acid is a much more important mass measure for controlling elevation of plasma homocysteine concentration.

Key Words: Methylenetetrahydrofolate reductase, Homocysteine, Folic acid, Vitamin B₁₂

Gynecol Obstet Reprod Med 2013;19:1-6

Introduction

Homocysteine is a thiol (SH)-containing amino acid, which is not used in protein synthesis but, instead, involved at the junction of two major metabolic pathways in human physiology.¹ It is an essential amino acid required for the growth of cells and tissues in the human body. The only source of homocysteine in the human organism comes from the methionine in dietary proteins which are mainly of animal origin. Methionine is the only essential, sulphur containing amino acid in mammalian diets. The interconversion of the two amino acids suggests that they share common regulatory mechanisms and metabolic functions. Homocysteine, in turn, uses two pathways for biotransformation: transsulphuration and remethylation.² First, in transsulphuration, it can be con-

densed with the amino acid serine in an irreversible sulphuration reaction catalysed by the enzyme cystathionine beta synthase (CBS) in the presence of pyridoxine (vitamin B₆) to form cystathionine, which is then itself reduced to form cysteine which can be excreted-thus allowing the body to dispose of sulphur-containing compounds. Alternatively, in the remethylation pathway, homocysteine can be reversibly methylated to re-form the essential amino acid methionine.¹ This pathway involves the methionine synthase (MS) enzyme which uses vitamin B₁₂ (cobalamin) as a cofactor and methylenetetrahydrofolate as the substrate. The formation of methylenetetrahydrofolate is catalysed by methylenetetrahydrofolate reductase (MTHFR), an enzyme that has an indirect, but important, influence on the remethylation of homocysteine to methionine, and which uses folic acid as a cofactor. Thus this reaction, which is essential for the recycling of folates from the methyltetrahydrofolate form, represents the intersection of folate and vitamin B₁₂ metabolism.² In the absence of or with reduced concentrations of vitamin B₆, of vitamin B₁₂ and of folic acid or with genetic metabolic defects, originating a failure in the metabolism of homocysteine or reduced dietary intake of B complex vitamins such as folic acid, B₆ and B₁₂ can cause hyperhomocysteinemia.^{1,2}

The deficit of MTHFR is the second most frequent hereditary defect of homocysteine metabolism.² The MTHFR gene has at least two functional polymorphisms, 677C-T and 1298A-C, either homozygous or heterozygous.³

¹Hacettepe University Faculty of Health Sciences, Department of Nutrition and Dietetics, Ankara

²Hacettepe University Faculty of Medicine, Department of Obstetrics and Gynecology, Ankara

³Hacettepe University Faculty of Medicine, Department of Biostatistics, Ankara

Address of Correspondence: Ç. Tuba Günebak Şahin
Hacettepe University Faculty of Health Sciences, Department of Nutrition and Dietetics, Ankara

Submitted for Publication: 21. 12. 2012

Accepted for Publication: 03. 04. 2013

The normal concentration of homocysteine in non-pregnant, healthy women of childbearing age is between 5.8 and 12.8 mmol/l. Levels of homocysteine are generally lower during pregnancy, either due to hormonal changes (physiological response to the pregnancy) or the normal increase in the glomerular filtration rate that accompanies pregnancy, increase in oestrogen, increasing demand for methionine by both the mother and the fetus and the uptake of homocysteine by the fetus and haemodilution.^{1,2,4-7}

There is cumulative evidence that hyperhomocysteinemia is an independent risk factor for coronary, cerebral and peripheral vascular diseases, psoriasis and some tumors.^{6,7} Although, over the last few years, homocysteine in general and increased homocysteine levels in particular have been associated with various complications during pregnancy, including defects of the neural tube and repeated miscarriages during the first 3 months, as well as intrauterine growth retardation (IUGR), preeclampsia, placental abruption, preterm delivery, congenital heart defects, Down syndrome, detachment of the placenta and fetal death in the second and third trimester of pregnancy.^{2,8} Both elevated total homocysteine (tHcy) and low folate and cobalamin status have been associated with recurrent miscarriage, neural tube defects, preterm birth, IUGR.⁸ Few studies have examined the extent to which elevated plasma homocysteine concentrations, associated with the MTHFR mutation, is modified by blood levels of nutritional cofactors for homocysteine metabolism.⁹ Based on these results, the objective of this study is to investigate the influence of MTHFR gene polymorphisms which may effect the interaction in between homocysteine, folic acid and vitamin B₁₂ metabolisms.

Material and Method

The subject of this study is consisted of 165 patients who were obtained from "Hacettepe University Maternal Fetal Medicine Data Base". These were married women of childbearing age with MTHFR gene polymorphisms who experienced bad obstetrical history (repeated fetal loss and/or perinatal complications) and undertook for medical examination to find out the etiology of previous bad pregnancy outcomes. Plasma total homocysteine, serum folic acid and vitamin B₁₂ levels of the subjects were analysed and the interrelation between these nutritional factors was examined. Inclusion criteria were consulted at Hacettepe University Adults' Hospital Gynaecology and Obstetrics Department, having diagnosis of MTHFR gene polymorphism, giving last birth in our hospital. Exclusion criteria were unwillingness to participate, being on treatment with drugs known to impair the absorption or utilization of folic acid or B₁₂ (e.g. phenytoin, antacids). Subjects were grouped according to subpolymorphism groups as MTHFR homozygous or heterozygous. Subjects with

MTHFR 677 C-T homozygous, MTHFR 1298 A-C homozygous and MTHFR compound heterozygous polymorphism were in the homozygous group. Subjects with MTHFR 677 C-T heterozygous, MTHFR 1298 A-C heterozygous polymorphism were in the heterozygous group. This study was approved by the Ethics Committee of Hacettepe University Faculty of Medicine according to World Medical Association Declaration of Helsinki (September 29th, 2009) and the Informed consent was obtained from each participant.

Data analyses were performed with Predictive Analysis Software (PASW), Statistic 18, IBM Company, 2009. Logarithmic transformation was applied to the variables and Pearson's correlation analysis was used to estimate the statistical correlation between serum folic acid and vitamin B₁₂ levels, homocysteine and folic acid levels. The correlation between plasma homocysteine level and serum folic acid level was also examined with Pearson correlation coefficient after applying logarithmic transformation. We adjusted serum folic acid levels for plasma homocysteine levels (<10.0 µmol/l or ≥10.01 µmol/l). Means of groups were compared with using one sample t test. Results were considered statistically significant at P<.05. We detected the significant difference of alteration of serum folic acid levels among MTHFR polymorphism groups with Mann-Whitney U test.

Means and Standard deviations (S.D.) (additionally minimum and maximum levels) were computed for plasma homocysteine level, serum folic acid and vitamin B₁₂ level as descriptive statistics. Frequency and percentage of MTHFR subpolymorphism groups were given as a nominal variable's descriptive statistics.

Results

Subjects' age ranged from 20 to 45 years with a mean age of 31,55±5,089 years. All subjects were MTHFR mutant (Table 1). Twenty-seven point nine percent (27,9%) of the subjects were MTHFR homozygous mutant (MTHFR 677 C-T homozygous, MTHFR 1298 A-C homozygous, MTHFR compound heterozygous) and 72,1 % of them were MTHFR heterozygous mutant (MTHFR 677 C-T heterozygous and MTHFR 1298 A-C heterozygous).

Table 1: MTHFR sub-polymorphism groups of subjects

MTHFR Polymorphism	n	%
677 C-T homozygous	21	12.7
677 C-T heterozygous	94	57.0
1298 A-C homozygous	8	4.8
1298 A-C heterozygous	25	15.1
Compound heterozygous	17	10.2
TOTAL	165	100.0

Plasma homocysteine, serum folate and vitamin B₁₂ levels of the subjects were 8.7 ± 4.7 $\mu\text{mol/l}$, 15.1 ± 14.7 mg/ml and 294.6 ± 127.0 pg/ml respectively (Table 2). Most of the subjects' (72,3%) plasma homocysteine level was below 10,0 $\mu\text{mol/l}$. Some of the subjects (35,5%) had high serum folic acid levels (>17 mg/ml) while 20,5% of the subjects' serum vitamin B₁₂ level was below 200 pg/ml . Subjects whose plasma homocysteine levels were below 10 $\mu\text{mol/l}$ had significantly higher serum folic acid levels ($p=0,02$). We found a statistically significant correlation between serum folic acid and vitamin B₁₂ levels of the subjects ($R=0,344$, $p<0,05$). Plasma homocysteine levels of the subjects were significantly correlated with serum folic acid levels ($R=0,248$, $p<0,05$).

Table 2: Plasma total homocysteine, serum folic acid and serum vitamin B₁₂ levels of the subjects

	X \pm SD	Minimum	Maximum
Homocysteine ($\mu\text{mol/l}$)	8.7 ± 4.7	3.4	53
Folic acid (mg/ml)	15.1 ± 14.7	4.1	191
Vitamin B ₁₂ (pg/ml)	294.6 ± 127.0	101.0	804.0

Subjects with MTHFR homozygous polymorphism had higher plasma homocysteine levels (Table 3). On the other hand, serum folic acid and vitamin B₁₂ levels of subjects with MTHFR heterozygous polymorphism were higher. MTHFR homozygous subjects' serum folic acid levels were significantly lower than the that of heterozygous subjects ($p<0.035$).

Discussion

Homocysteine is a non-protein-forming thiol containing amino acid derived from the demethylation of the essential amino acid methionine. Intracellular metabolism of homocysteine is regulated by two pathways, by which it is either further catabolised by transsulfuration to cysteine or remethylated to methionine. The transsulfuration pathway involves the enzyme CBS and requires vitamin B₆ as a cofactor. Remethylation to methionine is catalysed by MS, which requires folate in the form of 5-methyltetrahydrofolate as a co-substrate and vitamin B₁₂ in the form of methylcobalamin as a cofactor. MTHFR is a crucial folate dependent enzyme in the remethylation pathway, and is responsible for converting

5,10 methylenetetrahydrofolate to the cosubstrate 5-methyltetrahydrofolate.^{4,10} The deficit of this enzyme is the second most frequent hereditary defect of homocysteine metabolism.² The MTHFR gene has at least two functional polymorphisms, 677C-T and 1298A-C, either homozygous or heterozygous.³ In our study, most of the subjects (72,1%) were MTHFR heterozygous mutant (MTHFR 677 C-T heterozygous and MTHFR 1298 A-C heterozygous) and only 12,7% of the participants were 677 C-T homozygous.

In the absence of renal disease and hyperproliferative disorders, elevated levels of plasma homocysteine are generally a result of either a genetic defect in one of the enzymes involved in homocysteine metabolism or a nutritional deficiency of one of the vitamins that acts as a cofactor or co-substrate (folic acid, vitamin B₁₂ and vitamin B₆)⁴ Mild elevations in homocysteine, known as hyperhomocysteinemia, are associated with a common mutation in the MTHFR gene. This autosomal recessive mutation is a C-T substitution at base pair 677 resulting in an alanine to valine substitution and, as a consequence, in vivo enzyme activity is impaired. Individuals who are homozygous for the 677 C-T polymorphism tend to have elevated homocysteine levels when compared with individuals who are heterozygous for the mutation.⁴ Likewise, in our study, plasma total homocysteine level of the MTHFR homozygous subjects were higher than the heterozygous subjects. As mentioned in other studies, mainly elevated plasma homocysteine levels are observed in MTHFR 677 C-T homozygous subjects in our study. And, only 12,7% of the participants were from this sub-polymorphism group. The normal range of fasting homocysteine in adults has recently been 5-15 $\mu\text{mol/l}$.⁴ Dunkelgrun et al.¹¹ defined hyperhomocysteinemia as a fasting homocysteine level ≥ 15 $\mu\text{mol/l}$. However, the cut-off point set by the Nutrition Committee of the American Heart Association to define fasting hyperhomocysteinemia is ≥ 10 $\mu\text{mol/l}$.¹⁰ This cut-off point is also acceptable for pregnant women. In our study, mean plasma homocysteine level was found to be 8.7 ± 4.7 $\mu\text{mol/l}$ and most of the subjects' (72,3%) plasma homocysteine levels were below 10,0 $\mu\text{mol/l}$. As seen, most of the subjects were found to have normal plasma homocysteine concentration. But, at this point, it should be mentioned that these women were supplemented with folic acid

Table 3: Plasma total homocysteine, serum folic acid and serum vitamin B₁₂ levels of the subjects according to MTHFR polymorphism groups

MTHFR Polymorphism	Homozygous			Heterozygous		
	X \pm SD	Min	Max	X \pm SD	Min	Max
Homocysteine	$9,1 \pm 3,9$	3,9	22,0	$8,6 \pm 4,9$	3,4	53,0
Folic acid	$13,4 \pm 5,6$	4,1	24,0	$15,8 \pm 16,9$	6,1	191
Vitamin B ₁₂	$269,9 \pm 103,3$	101,0	562,0	$304,1 \pm 134,2$	118,0	804,0

and vitamin B₁₂ and homocysteine lowering effects of these two nutritional factors should not be undermined. People with the abnormal variant of MTHFR do not develop hyperhomocysteinemia if they have a relatively high intake of folate. This seems to be due to the methylation of folate in the intestinal mucosa during absorption; intestinal mucosal cells have a rapid turnover (some 48 h between proliferation in the crypts and shedding at the tip of the villus), and therefore it is not important that MTHFR is less stable than normal, as there is still an adequate activity of the enzyme in the intestinal mucosa to maintain a normal circulating level of methyltetrahydrofolate.¹² Similarly, vitamin B₁₂ is also an independent and significant nutritional factor that affects plasma homocysteine concentration. Several studies have demonstrated that vitamin B₁₂ may enhance the homocysteine-lowering effect of folic acid.^{1,2,4,8,10,13}

Plasma homocysteine level is accepted as a physiological indicator of folate level.¹⁴ MTHFR homozygous subjects, especially 677C-T homozygous, tend to have decreased concentrations of folate due to decreased enzyme activity. MTHFR homozygous subjects' serum folic acid levels were significantly lower than that of the heterozygous subjects ($p < 0.035$). Previous studies have shown that vitamin B₁₂ is also an important determinant for plasma homocysteine level, and that there was a similar interaction between the MTHFR genotype and vitamin B₁₂.⁹ Similarly, in our study, serum vitamin B₁₂ levels of the MTHFR homozygous subjects were lower than that of the MTHFR heterozygous subjects. An inverse association between vitamin B₁₂ and homocysteine levels is also plausible because vitamin B₁₂ is a cofactor for MS transforming homocysteine to methionine, which is directly linked to the remethylation cycle. Vitamin B₁₂ levels happened to be lower among individuals with homozygous genotype for the MTHFR mutation than among those with other genotypes.⁹ In addition to vitamin B₁₂, several studies have shown an inverse association between blood homocysteine levels with plasma or serum levels of folate, vitamin B₆.¹⁵ Likewise, in our study, subjects whose plasma homocysteine levels were below 10 $\mu\text{mol/l}$ had significantly higher serum folic acid levels ($p = 0.02$). In this point of view, plasma homocysteine concentration of 10 $\mu\text{mol/l}$ can be accepted as a cut-off point. Cheng et al have indicated that serum folate, vitamin B₁₂ and/or MTHFR 677C-T mutation significantly correlated with plasma fasting homocysteine concentration. Although low serum folate plays an important role in determining fasting homocysteine concentration.¹⁰ We found a statistically significant correlation between serum folic acid and vitamin B₁₂ levels of the subjects ($R = 0.344$, $p < 0.05$). Plasma homocysteine levels of the subjects were significantly correlated with serum folic acid levels ($R = 0.248$, $p < 0.05$).

Hague¹ mentioned that with the recognition of hyperho-

mocysteinaemia, it is essential to establish the efficacy and safety of folic acid supplementation in the prophylaxis of recurrent disease in such women before such treatment is recommended for routine clinical practice. It is effective to reduce the homocysteine level and high homocysteine level associated with pregnancy disorders with folic acid and vitamin B₁₂.⁹ It is estimated that there can be a 25% reduction in homocysteine levels with supplementation of 0.5 - 5.7 mg/day of folate.¹⁵ MTHFR mutant individuals have a high folate requirement and pregnant women with this polymorphism are at an increased risk of folate deficiency as a result of increased folate catabolism and utilisation. For preventing the fetus against occurrence of neural tube defects, national expert committees advise the current recommendations: 4-5 mg/d folic acid in tablet form for the prevention of neural tube defect recurrence; 400 $\mu\text{g/d}$ folic acid to be commenced before conception and continued until the twelfth week of pregnancy for the prevention of first occurrence.⁴ In such a manner, we are supplementing pregnant women with 5 mg/week folic acid from 3 months before pregnancy till the end of pregnancy. Supplementation with vitamin B₁₂ is also recommended.¹⁶ High intakes of vitamin B₁₂ may compensate for the effect of the MTHFR mutation by accelerating transformation of homocysteine to methionine even when MTHFR activity is reduced.⁹ van Horn et al reported that in patients with a vitamin B₁₂ deficiency who may have intermediate or severe hyperhomocysteinemia, B₁₂ can normalize homocysteine levels in 70% of the cases.¹⁵ It is found that the association between the MTHFR genotype and homocysteine levels was the strongest among individuals with lower folate and lower vitamin B₁₂ levels while a moderate association was observed among individuals with higher folate and lower vitamin B₁₂ levels.^{9,17} Deshmukh et al¹⁷ found that both 2 and 10 $\mu\text{g/d}$ of oral vitamin B₁₂ (cyanocobalamin) significantly reduced plasma tHcy concentrations in otherwise healthy, free-living, rural participants and reported that daily oral supplementation with physiological doses of vitamin B₁₂ is an effective community intervention to reduce tHcy. In coordination with these authors, we are supplementing women whom we consulted with 10 $\mu\text{g/d}$ vitamin B₁₂ in the form of cyanocobalamin. Several intervention studies have demonstrated that supplementation with folate or the combined supplementation with folate and vitamin B₁₂ effectively reduce plasma homocysteine levels.⁹ Supplementation by folate showed a larger decrease in plasma homocysteine levels among individuals with the homozygous C677T mutation than among those without it. Except for our study, no trial has examined which of folate, vitamin B₁₂ or their combination is most effective for the reduction of homocysteine levels according to the MTHFR genotype. According to our results, folate is the most effective nutritional factor for the reduction of homocysteine level.

The balance of methionine and homocysteine in the body can be disturbed because of inadequate intake of three B-vitamins, or cause genetic defects leading to low or absent enzyme activity rates in the relevant metabolic pathways. Several investigators have conducted studies examining the effect on homocysteine levels in response to changes in folic acid intake. The results confirmed that folic acid supplements decrease homocysteine levels at all levels of homocysteine, but that the amount of reduction was greater for higher baseline levels, and lower blood folate levels.¹⁸

Conclusion

In conclusion, elevated total homocystein concentration has been associated with several pregnancy complications and bad obstetrical history, in MTHFR mutant pregnant women. In the same perspective, other factors which have been found to be related with pregnancy complications, especially recurrent miscarriages, are low folic acid and cobalamin concentrations. Pregnant women with total homocysteine concentration of $\geq 10 \mu\text{mol/l}$ should be evaluated for MTHFR polymorphism. Especially, MTHFR homozygous mutant individuals are more prone to higher total homocysteine and lower folic acid levels. Both folic acid and vitamin B₁₂ are important and effective factors in homocysteine metabolism in MTHFR mutant subjects. But, folic acid is a much more important mass measure for controlling elevation of plasma homocysteine concentration. Because of this, both folic acid and vitamin B₁₂ levels of these patients should also be assessed along with homocysteine concentrations, but more importantly folic acid levels should be considered. In addition to this, supplementing these patients with folic acid and vitamin B₁₂ can be effective for controlling total homocysteine concentration. In the order of importance, total homocysteine, folic acid and vitamin B₁₂ levels of a patient with bad obstetrical history can give an idea about existence of MTHFR polymorphism. In that respect, analysing total homocysteine level and folic acid and vitamin B₁₂ levels, before giving decision of analysing MTHFR polymorphism, can be much more cost effective.

Metilentetrahidrofolat Redüktaz Enzim

Mutasyonları ve Homosistein, B₁₂ Vitamini ve Folat Kan Düzeylerinin İlişkisi

AMAÇ: Bu çalışmanın amacı homosistein, folik asit ve B₁₂ vitamini metabolizmaları arasındaki etkileşimi etkileyebilen MTHFR gen polimorfizminin etkisini araştırmaktır.

GEREÇ VE YÖNTEM: Bu çalışmaya dahil edilen 165 hastaya "Hacettepe Üniversitesi Maternal Fetal Tıp Veri Tabanı"ndan ulaşılmıştır. Bireyler kötü obstetrik öyküsü olan, MTHFR gen polimorfizmi taşıyan, doğurganlık çağındaki evli kadınlardır. Bireylerin plazma total homosistein, serum folik asit ve B₁₂ vi-

tamini düzeyleri analiz edilmiş ve bu beslenme faktörleri ile arasındaki ilişki araştırılmıştır.

BULGULAR: MTHFR mutasyonu olan bireylerde, homosistein metabolizmasında, hem folik asit hem de B₁₂ vitamini istatistiksel olarak anlamlı şekilde önemli ve etkilidirler.

SONUÇ: Folik asit, plazma homosistein konsantrasyonunda meydana gelebilen artışın kontrolünde çok daha önemli bir parametredir.

Anahtar Kelimeler: Metilentetrahidrofolat redüktaz, Homosistein, Folik asit, B₁₂ vitamini

REFERENCES

1. Hague WM. Homocysteine and pregnancy. *Best Pract Res Clin Obstet Gynaecol* 2003;17:459-69.
2. de la Calle M. et al. Homocysteine, folic acid and B-group vitamins in obstetrics and gynaecology. *Eur J Obstet Gynecol Reprod Biol* 2003;107:125-34.
3. Brustolin SR. Giugliani, and T.M. Felix Genetics of homocysteine metabolism and associated disorders. *Braz J Med Biol Res* 2010;43:1-7.
4. Holmes VA. Changes in haemostasis during normal pregnancy: does homocysteine play a role in maintaining homeostasis? *Proc Nutr Soc* 2003;62:479-93.
5. Bottiglieri T. Homocysteine and folate metabolism in depression. *Prog Neuropsychopharmacol Biol Psychiatry* 2005;29:1103-12.
6. Deminice R. et al. Effects of a low-protein diet on plasma amino acid and homocysteine levels and oxidative status in rats. *Ann Nutr Metab* 2009;54:202-7.
7. Medina M. Urdiales JL. Amores-Sánchez MI. Roles of homocysteine in cell metabolism: old and new functions. *Eur J Biochem* 2001;268:3871-82.
8. Varela-Moreiras G. Murphy M.M. Scott J.M. Cobalamin, folic acid, and homocysteine. *Nutr Rev* 2009; 67 Suppl 1,69-72.
9. Moriyama Y. et al. Effects of serum B vitamins on elevated plasma homocysteine levels associated with the mutation of methylenetetrahydrofolate reductase gene in Japanese. *Atherosclerosis* 2002;164:321-8.
10. Cheng CH. et al. B-vitamins homocysteine and gene polymorphism in adults with fasting or post-methionine loading hyperhomocysteinemia. *Eur J Nutr* 2008;47:491-8.
11. Dunkelgrun M. et al. Methionine loading does not enhance the predictive value of homocysteine serum testing for all-cause mortality or major adverse cardiac events. *Intern Med J* 2009;39:13-8.
12. Bender D. The Vitamins. In: *Introduction to Human Nutrition*. (V.H. Gibney MJ, Kok FJ, ed.) 2002; pp.125-

- 176, Blackwell Publishing, Oxford, UK.
13. Kumar J. et al. Homocysteine levels are associated with MTHFR A1298C polymorphism in Indian population. *J Hum Genet* 2005;50:655-63.
 14. Ozturk O. Karaer S. Uncu D. et al. Serum homocysteine, folate and vitamin B12 levels in pregnant and non-pregnant women. *J Med Sci* 2006;26:121-5.
 15. van Horn L.A.S. Thedford K. Baltes A. Other Dietary Components and Cardiovascular Risk. In: *Nutrition in the Prevention and Treatment of Disease* (R.C. Coulston AM, Monsen ER, ed.) 2001; p. 291-302, Academic Press, San Diego, California.
 16. Roche H. Mensink RP. Molecular Aspects of Nutrition. In: *Nutrition and Metabolism* (M.I. Gibney MJ, Roche HM, ed.) 2003; p. 6-29, Blackwell Publishing, Oxford, UK.
 17. Deshmukh US. et al. Effect of physiological doses of oral vitamin B12 on plasma homocysteine: a randomized, placebo-controlled, double-blind trial in India. *Eur J Clin Nutr* 2010;64:495-502.
 18. Beresford S. Boushey C.J. Homocysteine, Folic Acid, and Cardiovascular Disease Risk. In: *Preventive Nutrition-The Comprehensive Guide for Health Professionals* (A. Bendich, Deckelbaum, RJ, ed.) 2001; 191-219, Humana Press: Totowa, New Jersey, USA.