

# Effect of genetic polymorphisms involved in folate metabolism on the concentration of serum folate and plasma total homocysteine (p-tHcy) in healthy subjects after short-term folic acid supplementation: a randomized, double blind, crossover study

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**Abstract** Data on the effect of combined genetic polymorphisms, involved in folate metabolism, on the concentration of serum folate after folic acid supplementation are scarce. Therefore, we investigated the impact of seven gene polymorphisms on the concentration of serum folate and p-tHcy in healthy subjects after short-term folic acid supplementation. In a randomized, double blind, crossover study, apparently healthy subjects were given either 0.8 mg folic acid per day ( $n = 46$ ) or placebo ( $n = 45$ ) for 14 days. The washout period was 14 days. Fasting blood samples were collected on day 1, 15, 30 and 45. Data on subjects on folic acid supplementation ( $n = 91$ ) and on placebo ( $n = 45$ ) were used for the statistical analysis. The

concentration of serum folate increased higher in subjects with higher age ( $53.5 \pm 7.0$  years) than in subjects with lower age ( $24.3 \pm 3.2$  years) after folic acid supplementation ( $p = 0.006$ ). The baseline concentration of serum folate in subjects with polymorphism combination, reduced folate carrier protein, RFC1-80 GA and methylenetetrahydrofolate reductase, MTHFR677 CT+TT, was lower than RFC1-80 AA and MTHFR677 CT+TT ( $p = 0.002$ ). After folic acid supplementation, a higher increase in the concentration of serum folate was detected in subjects with polymorphism combination RFC1-80 GA and MTHFR677 CC than RFC1-80 GG and MTHFR CT+TT combination ( $p < 0.0001$ ). The baseline concentration of plasma total homocysteine (p-tHcy) was altered by combined polymorphisms in genes associated with folate metabolism. After folic acid supplementation, in subjects with combined polymorphisms in methylenetetrahydrofolate dehydrogenase, MTHFD1-1958 and MTHFR-677 genes, the concentration of p-tHcy was changed ( $p = 0.002$ ). The combination of RFC1-80 and MTHFR-677 polymorphisms had a profound affect on the concentration of serum folate in healthy subjects before and after folic acid supplementation.

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## Introduction

Folate deficiency increases the risk of cardiovascular diseases (CVD), certain types of cancer and possibly decline in cognitive abilities (Daly et al. 1995; Shane 2010).

Maternal folate deficiency and genetic polymorphisms in folate metabolism have also been linked to neural tube defects (NTDs) in new born babies (Etheredge et al. 2012). An attempt to reduce the prevalence of NTDs in the USA, the fortification of wheat flour with folic acid was started in 1998 (US FDA 1996). Furthermore, folic acid supplementations have been used to reduce the concentration of plasma total homocysteine (p-tHcy) in patients with hyperhomocysteinemia, which has been associated with CVD (Finch and Joseph 2010).

Folic acid is transported in the intestinal cells by a proton-coupled folate transporter (PCFT) (Zhao et al. 2011; Yee et al. 2010). Mostly in liver, intracellular folic acid is first reduced to dihydrofolate and later to tetrahydrofolate (THF) by the enzyme dihydrofolate reductase (DHFR). The enzyme serine hydroxymethyltransferase (SHMT) converts THF into methylenetetrahydrofolate (methylene-THF), which is reduced to 5-methyl-THF by methylenetetrahydrofolate reductase (MTHFR). Vitamin B<sub>12</sub> and 5-methyl-THF participate in remethylation of homocysteine into methionine by methionine synthase (MTR) (Daly et al. 1995; Shane 2010). Dietary folates do not enter directly into the intestinal cells. The enzyme  $\gamma$ -glutamylhydrolase (GH) removes glutamate residues from the polyglutamates and generates folate monoglutamates, which are transported in the cells by a reduced folate carrier 1 (RFC1). RFC1 mediates bidirectional movement of folates across the cell membrane; therefore, an efficient RFC1 may maintain an optimal concentration of intracellular and extracellular folates (Zhao et al. 2011; Yee et al. 2010). Intracellular folate monoglutamates are topped with glutamate to form polyglutamates by the enzyme folylpolyglutamate synthase (FPGS). The newly formed polyglutamates are trapped and stored in the cells (Daly et al. 1995; Shane 2010).

Intracellular folates are essential for the synthesis of thymidylate; the reaction is performed by the enzyme thymidylate synthase (TS). The enzyme, methylene-THF-dehydrogenase1 (MTHFD1), provides 5, 10-methylene-THF and 10-formyl-THF, which are involved in the synthesis of DNA. Folate is also involved in the conversion of serine to glycine, where serine provides one-carbon units for the folate-dependent one-carbon reactions (Daly et al. 1995; Shane 2010).

Previously, it had been reported that individual polymorphisms in MTHFR-1298A>C and MTHFR-1317T>C did not have any effect on the concentration of p-tHcy; however, subjects with MTHFR-677 TT had significantly higher concentrations of p-tHcy (Pereira et al. 2004; Devlin et al. 2006; Zappacosta et al. 2014; Yang et al. 2008; Anderson et al. 2013). It was also reported that folic acid intake (150  $\mu$ g/day) in subjects with MTHFR677 TT had a counter effect and reduced the concentration of p-tHcy

(Yang et al. 2008). Moreover, subjects with interaction between MTHFR-677 TT and RFC1-80 GG had increased concentration of p-tHcy (Devlin et al. 2006). In women, with unexplained recurrent miscarriages, 0.5 mg folic acid supplementation caused a significant reduction in the concentration of p-tHcy; however, the effect was pronounced in women with the highest baseline concentration of p-tHcy and MTHFR-677-TT polymorphism (Nelen et al. 1998).

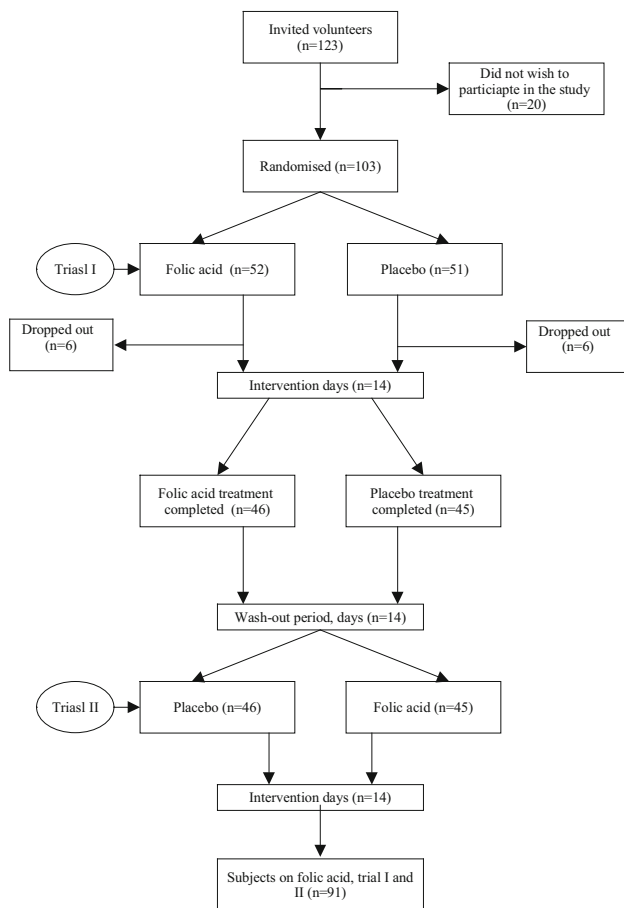
Most studies have reported the effect of individual polymorphisms on the concentration of p-tHcy and serum folate, besides the reported data are not consistent. Furthermore, data on the combined effect of gene polymorphisms, in folate metabolizing genes, on the concentration of serum folate and p-tHcy after folic acid supplementation are scarce (Pereira et al. 2004; Devlin et al. 2006; Zappacosta et al. 2014; Yang et al. 2008; Anderson et al. 2013; Nelen et al. 1998; DeVos et al. 2008). Therefore, in a crossover study, we assessed the combined effect of MTHFR-677C>T, MTHFR-1298A>C, RFC1-80G>A, MTHFD1-1958G>A, MTR-2756A>G, DHFR-19 del and TS gene polymorphisms on the concentration of serum folate and p-tHcy after short-term folic acid supplementation in healthy subjects.

## Materials and methods

### Study design and study population

A randomized, double blind, crossover study.

Flow chart diagram of study participants is provided (Fig. 1). Inclusion criteria were healthy male and female subjects, who were not using any drugs, medications or vitamin supplementations. Individuals with cardiovascular disease, cancer and diabetes were not included in this study. Women who were pregnant were not included in this study. All subjects were ethnic Norwegian. The participants reported that they were not suffering from any disease. Based on their self-reported health evaluation, apparently healthy employees and students ( $n = 103$ ) from University of Agder, Kristiansand, Norway, agreed to participate in the study (Fig. 1). One hundred and three subjects were randomized for supplementation. During the first supplementation trial, they were given either folic acid (0.8 mg folic acid/per day) ( $n = 52$ ) or placebo ( $n = 51$ ) for a period of 14 days. Twelve subjects dropped out, and 91 subjects completed the study (Fig. 1). Fasting blood samples were collected before and after the supplementation period. Individuals who received folic acid during the first trial received placebo during the second trial and vice versa. Washout period between the two trials was 14 days. All subjects provided fasting blood sample in the morning



**Fig. 1** Flow chart diagram shows the steps taken for the collection of blood samples from healthy subjects before and after supplementation with folic acid and placebo

(between 7 am and 9 am) on day 1 (1 day before folic acid supplementation), day 15, day 30 and day 45. Blood samples were placed on the ice in the dark. Blood was used for the isolation of DNA. Serum and plasma were isolated from the blood samples and were stored at  $-80^{\circ}\text{C}$  until all biochemical and molecular biology analyses were performed. Data from subjects on folic acid supplementation ( $n = 91$ ) and subjects on placebo ( $n = 46$ ) were used for statistical analyses.

Dietary intake data were collected during the first trial; the data were available from 96 subjects, who had also provided the blood samples at the first meeting. Findings on the dietary intake data were reported previously (Hernes et al. 2012).

We have reported previously that folic acid supplement, 0.6 mg folic acid, given to healthy individuals increased the concentration of serum folate significantly after 5 weeks (Mansoor et al. 1999). Based on these observations, we calculated the sample size based on 80 % power.

The present study was performed according to the guidelines established in Declaration of Helsinki. The

study protocol was approved by the Regional Committee of Ethics, Oslo and Data Inspectorate in Norway.

## Genotyping

Briefly, reaction volume 20  $\mu\text{L}$  contained 1 $\times$  Amplitaq Gold reaction buffer, 500  $\mu\text{M}$  dNTP, 0.5  $\mu\text{M}$  of each primer (primer sequences are provided in Table 1), 1.25 U Amplitaq Gold DNA polymerase (Life Technologies Inc.), 3 mM  $\text{MgCl}_2$  for all polymorphisms except DHFR-19del and TS, where 1.5 mM  $\text{MgCl}_2$  was used and 200 ng DNA. PCR profile was 95  $^{\circ}\text{C}$ , 5 min, followed by 35 cycles of 95  $^{\circ}\text{C}$ , 30 s, the annealing temperature as specified in Table 1, 30 s, 72  $^{\circ}\text{C}$ , 30 s and final extension incubation at 72  $^{\circ}\text{C}$  for 7 min. Some of the polymorphisms were analyzed by cutting 10  $\mu\text{l}$  PCR products with restriction enzyme with the corresponding buffer, at 37  $^{\circ}\text{C}$ , overnight. The products, after PCR and cutting, were analyzed by the agarose gel electrophoresis (Fredriksen et al. 2007; Charasson et al. 2009; Frosst et al. 1995; Markan et al. 2007; Gemmati et al. 2009; Horie et al. 1995; Skibola et al. 2002; Canalle et al. 2006). In DHFR-19 del, where a 19 nucleotide deletion (D) in intron 1 or wild type, no deletion (W) was detected. In TS, doublets (2R) or triplets (3R) of an element of 28 nucleotides in the promoter of the gene were detected. The resulting product sizes for the gene polymorphisms are given in Table 1. Details of the PCR methods have been provided previously (Cabo et al. 2015).

## Measurement of folate and vitamin B<sub>12</sub> in the serum samples

The concentrations of serum folate and serum vitamin B<sub>12</sub> were measured by a Cobas 6000 in the laboratory of Medical Biochemistry, Sørlandet Hospital HF, Arendal, Norway. The coefficient for variation (CV) for the assays for serum folate and vitamin B<sub>12</sub> was <10 %.

## Measurement of p-tHcy

The concentration of p-tHcy was measured by an ion-pair high-performance liquid chromatography (HPLC) method with a fluorescence detector (Mansoor et al. 1992). The HPLC method had a CV < 8 % for the p-tHcy measurements.

## Statistical methods

Data are presented as mean and standard deviation ( $\pm\text{SD}$ ). The concentrations of serum folate and p-tHcy had not normal distribution (Gaussian distribution curve); therefore, we used the log-converted values of serum folate and p-tHcy in statistical analyses (Mansoor et al. 1995). We

**Table 1** PCR methods used in this study

Gene	Primers	Annealing temperature in PCR (°C)	Restriction enzyme	PCR product (bp)	References
MTHFR-677C>T	(S) TGAAGGAGAAGGTGTCTGCGGGA (AS) AGGACGGTGCGGTGAGAGTG	55	Hinf	198, 175, 23	Fredriksen et al. (2007), Charasson et al. (2009), Frosst et al. (1995), Markan et al. (2007)
MTHFR-1298A>C	(S) CTTTGGGGAGCTGAAGGACTACTA (AS) CACTTTGTGACCATTCCGGTTTG	55	MboII	84, 56, 31, 30, 28, 18	Fredriksen et al. (2007), Charasson et al. (2009), Frosst et al. (1995), Markan et al. (2007)
RFC1-80G>A	(S) AGTGTACACCTTCGTCCCTC (AS) CTCCCGCGTGAAGTTCTT	55	CfoI	162, 125, 68, 37	Fredrikson et al. (2007), Markan et al. (2007)
MTHFD1-1958G>A	(S) CACTCCAGTGTGTTGCCATG (AS) GCATCTTGAGAGCCCTGAC	55	MspI	267, 196, 70, 56, 8	Fredriksen et al. (2007), Charasson et al. (2009), Markan et al. (2007)
MTR-2756A>G	(S) TGTTCCCAGCTGTTAGATGAAAAT (AS) GATCCAAAGCCTTTTACACTCCTC	55	HaeIII	211, 131, 80	Fredriksen et al. (2007), Charasson et al. (2009)
DHFR-19del	(F1) CCACGGTCGGGGTACCTGGG (F2) ACGGTCGGGGTGGCCGAC (R) AAAAGGGGAATCCAGTCGG	63		113, 92	Gemmati et al. (2009), Horie et al. (1995), Skibola et al. (2002), Canalle et al. (2006)
TS	(P1) GTGGCTCCTGCGTTTCCCCC (P2) CCAAGCTTGGCTCCGAGCCGG CCACAGGCATGGCGCGG	62			Gemmati et al. (2009), Horie et al. (1995), Skibola et al. (2002), Canalle et al. (2006)

*MTHFR* methylene tetrahydrofolate reductase, *RFC1* reduced folate carrier protein-1, *MTHFD 1* methylenetetrahydrofolate dehydrogenase 1, *MTR* methionine synthase, *DHFR* dihydrofolate reductase, *TS* thymidylate synthase

tested differences between two groups by Student t test, and the differences between more than two groups were estimated by ANOVA. *p* values <0.05 were considered significant. We used ANOVA post hoc Bonferroni test for multiple comparisons to explore the effect of genotype groups on the concentration of serum folate and p-tHcy. In this test, *p* value required for statistical significance was much lower than 0.05. Age and BMI were compared between the groups by a nonparametric Mann–Whitney *U* test. Kruskal–Wallis test was used for testing comparison between three parameters. Hardy–Weinberg equilibrium (HWE) was examined with Chi-square test. We used Statview 4.5 for the Macintosh for the statistical analyses.

## Results

The characteristics of the study participants and the concentrations of serum folate and p-tHcy are reported in

Table 2. The concentration of serum folate increased and the concentration of p-tHcy decreased after folic acid supplementation ( $p < 0.0001$  and  $p = 0.0001$ ); an increase in the concentration of serum folate was  $23.0 \pm 30.6$  nmol/L (mean  $\pm$  SD) and a decrease in the concentration of p-tHcy was  $0.7 \pm 1.4$   $\mu$ mol/L (mean  $\pm$  SD) (Table 2). The baseline concentration of serum folate in the employees was not significantly higher than in the students ( $p = 0.2$ ). However, the concentration of serum folate increased significantly higher in the employees than in the students ( $p = 0.006$ ). Only one subject had hyperhomocysteinemia; the p-tHcy concentration was  $>15$   $\mu$ mol/L. None of the study subjects had deficiency of serum folate. Allele frequency for genes has been tested for Hardy–Weinberg equilibrium and is provided in Table 3.

The subjects on folic acid supplementation had higher concentration of serum folate after washout and placebo periods than the baseline concentrations  $22.04 \pm 6.67$  versus  $18.05 \pm 6.45$  nmol/L (mean  $\pm$  SD) ( $p < 0.001$ ).

**Table 2** Age and BMI of the study subjects and the concentrations of serum folate and p-tHcy before and after folic acid supplementation

	All	Employees	Students
Folic acid group, number	91	49	42
Age, years	53.5 ± 7.0	24.3 ± 3.2* <sup>1</sup>	
BMI		24.7 ± 3.3	23.9 ± 3.6
Sex, female/male ratio	71/24		
Serum folate, nmol/L	17.2 ± 6.3	18.0 ± 4.8	16.3 ± 5.5
Serum folate, post-intervention	40.1 ± 30.7* <sup>1</sup>	48.1 ± 38.7* <sup>2</sup>	30.6 ± 11.1* <sup>3</sup>
p-tHcy, μmol/L	8.4 ± 3.5	8.6 ± 2.3	8.2 ± 4.5
p-tHcy, post-intervention	7.8 ± 3.0* <sup>2</sup>	8.2 ± 2.2* <sup>4</sup>	7.4 ± 3.8* <sup>5</sup>
Placebo group, number	46		
Serum folate, nmol/L	16.5 ± 5.6		
Serum folate, post-intervention	17.5 ± 6.3* <sup>6</sup>		
p-tHcy, μmol/L	8.8 ± 4.3		
p-tHcy, post-intervention	8.6 ± 2.9		
Female/male ratio	71/24		

Data are presented as mean ± SD. Log-converted values of serum folate and p-tHcy were used in the analyses

\*<sup>1</sup>  $p < 0.0001$ , \*<sup>2</sup>  $p < 0.0001$ , \*<sup>3</sup>  $p < 0.0001$ , \*<sup>4</sup>  $p = 0.009$ , \*<sup>5</sup>  $p = 0.007$ , \*<sup>6</sup>  $p = 0.01$ , #<sup>1</sup> baseline concentrations versus after supplementation— $p < 0.0001$ , #<sup>2</sup> baseline concentration versus after treatment— $p = 0.0001$

Therefore, the combined data on folic acid supplementation from trial I and trial II ( $n = 91$ ) and placebo treatment data from trial I ( $n = 45$ ) were used in the statistical analysis.

There was a significant difference in the concentration of serum folate and p-tHcy in individuals with MTHFR677CC, CT and TT genotypes after folic acid supplementation ( $p = 0.01$  and  $p = 0.0006$ ) (Table 3).

We combined homozygote (wild type), heterozygote and homozygote (variant type) for MTHFR-677C>T versus MTHFR-1298A>C, RFC1-80G>A, MTHFD1-1958G>A, MTR-2756A>G, DHFR-19 del and TS polymorphisms into groups. The effect of polymorphism combinations on the concentrations of serum folate and p-tHcy was explored by multiple comparisons.

The baseline concentration of serum folate was significantly different in RFC1-80 polymorphism combinations alone ( $p = 0.03$ ) Table 4. The concentration was highest in RFC1-AA and MTHFR677 CT and TT than other polymorphism combinations ( $p = 0.002$ ) (Table 4).

The increase in the concentration of serum folate was significantly different in different genotype combinations,

MTHFR677 versus MTHFR1298, MTHFD11958, MTR2756, TS and RFC1-80 ( $p = 0.02$ ,  $p = 0.03$ ,  $p = 0.03$ ,  $p = 0.04$  and  $p = 0.0003$ ) (Table 4).

The concentration of serum folate in MTHFR1298AC and CC and MTHFR677CC combination was significantly higher than MTHFR1298AC and CC and MTHFR677CT and TT combination ( $p = 0.005$ ). MTHFD1-1958GA and AA and MTHFR677CC also showed a significant difference in the concentration of serum folate than MTHFD1-1958GG and MTHFR677CT and TT ( $p = 0.003$ ). Similar observations were done for TS-2R2R and TS-2R3R and MTHFR677CC versus TS-2R2R and TS-2R3R and MTHFR677CT and TT ( $p = 0.008$ ). The most significant differences were observed for RFC1GA and MTHFR677CC versus RFC1GG and MTHFR677CT and TT and RFC1GA, MTHFR677CC versus RFC1GA and MTHFR677CT and TT ( $p < 0.0001$  and  $p = 0.0002$ ) (Table 4).

In Table 5, we could show that the baseline concentration of p-tHcy was significantly different in combined polymorphisms, MTHFR677 versus MTHFR1298, MTHFD1-1958, MTR2756, DHFR19Del, TS and RFC1-80 ( $p = 0.002$ ,  $p = 0.007$ ,  $p = 0.007$ ,  $p = 0.006$ ,  $p = 0.006$  and  $p = 0.01$ ). The combination MTHFR1298AA and MTHFR677CC polymorphisms had lower p-tHcy concentration than MTHFR1298AA and MTHFR677CT and TT ( $p = 0.0003$ ). The MTHFD1-1958GA and AA and MTHFR677CC had also significantly lower concentration of p-tHcy than MTHFD1-1958GA and AA and MTHFRCT and TT ( $p = 0.004$ ). MTR2756AG and GG and MTHFR677CC versus MTR2756AG and GG and MTHFR677CT and TT had difference in the concentration of p-tHcy (0.004). Similar observations were done for two different combinations of DHFR19Del and MTHFR677 ( $p = 0.002$  and  $p = 0.002$ ), one for TS and MTHFR677 ( $p = 0.008$ ) and one for RFC1-80 and MTHFR677 ( $p = 0.001$ ) (Table 5).

The concentrations of serum vitamin B<sub>12</sub> and p-tHcy were significantly higher in individuals with MTHFR-677C>T-TT than in individuals with CC and CT polymorphisms ( $p < 0.05$  and  $p < 0.05$ ).

Vitamin B<sub>12</sub>, vitamin B<sub>2</sub> and protein intakes were significantly different in individuals with MTHFR-1298A>C polymorphisms ( $p < 0.05$ ,  $p < 0.05$  and  $p < 0.05$ , respectively). Similarly, vitamin B<sub>12</sub> intake was significantly different in individuals with DHFR-19del polymorphism ( $p < 0.05$ ) (Table 6).

## Discussion

The baseline concentrations of serum folate and p-tHcy in the study subjects are in accordance with the previously



**Table 3** Concentrations of serum folate and p-tHcy after folic acid supplementation according to the genotypes

	Serum folate over baseline and after supplementation				p-tHcy over baseline and after supplementation		
	Allele frequency	Baseline	After supplementation	<i>p</i> value	Baseline	After supplementation	<i>p</i> value
<i>MTHFR-677C&gt;T</i>							
CC ( <i>n</i> = 40)	C:0.67	18.0 ± 6.2	49.5 ± 40.1*	<0.0001	7.3 ± 1.9	7.0 ± 1.4**	0.22
CT ( <i>n</i> = 43)	T:0.33	16.4 ± 5.8	34.6 ± 20.0	<0.0001	8.7 ± 2.3	7.8 ± 2.0	<0.0001
TT ( <i>n</i> = 8)		17.8 ± 9.0	26.0 ± 9.4	0.005	12.6 ± 8.9	11.7 ± 7.6	0.32
<i>MTHFR-1298A&gt;C</i>							
AA ( <i>n</i> = 46)	A:0.69	17.9 ± 6.5	35.5 ± 19.3	<0.0001	8.9 ± 4.5	8.2 ± 3.9	0.01
AC ( <i>n</i> = 34)	C:0.31	16.7 ± 6.3	43.2 ± 35.7	<0.0001	8.2 ± 2.0	7.5 ± 1.5	0.007
CC ( <i>n</i> = 11)		15.7 ± 5.4	50.3 ± 48.8	0.0003	7.3 ± 1.7	7.0 ± 1.6	0.3
<i>RFC1-80G&gt;A</i>							
GG ( <i>n</i> = 26)	G:51	16.6 ± 5.1	30.8 ± 11.4	<0.0001	8.1 ± 1.9	7.4 ± 1.4	0.04
GA ( <i>n</i> = 41)	A:49	16.3 ± 6.0	41.6 ± 33.3	<0.0001	8.7 ± 4.6	8.0 ± 3.8	0.02
AA ( <i>n</i> = 24)		19.4 ± 7.5	47.4 ± 37.6	<0.0001	8.3 ± 2.5	7.8 ± 2.4	0.02
<i>MTHFD1-1958G&gt;A</i>							
GG ( <i>n</i> = 32)	G:0.58	16.9 ± 6.2	37.8 ± 29.5	<0.0001	8.3 ± 1.8	7.5 ± 1.4	0.3
GA ( <i>n</i> = 41)	A:0.42	16.4 ± 4.6	41.0 ± 35.0	<0.0001	8.6 ± 4.7	8.1 ± 4.0	0.02
AA ( <i>n</i> = 18)		19.7 ± 9.0	42.0 ± 21.2	<0.0001	8.3 ± 2.5	7.6 ± 2.2	0.003
<i>MTR-2756A&gt;G</i>							
AA ( <i>n</i> = 54)	A:0.78	17.3 ± 6.5	43.2 ± 35.2	<0.0001	8.3 ± 2.3	7.7 ± 1.9	0.008
AG ( <i>n</i> = 34)	G:0.22	17.4 ± 6.1	35.4 ± 22.1	<0.0001	8.5 ± 4.9	7.7 ± 4.2	0.005
GG ( <i>n</i> = 3)		15.0 ± 4.1	35.1 ± 15.8	0.02	9.7 ± 5.5	9.6 ± 5.0	0.9
<i>DHFR-19del</i>							
WW ( <i>n</i> = 34)	W:0.59	16.3 ± 5.2	42.3 ± 35.6	<0.0001	8.4 ± 2.2	7.7 ± 1.7	0.06
WD ( <i>n</i> = 36)	D:0.41	17.7 ± 6.9	34.0 ± 22.0	<0.0001	8.6 ± 4.9	8.1 ± 4.4	0.14
DD ( <i>n</i> = 21)		18.0 ± 6.8	46.3 ± 33.5	<0.0001	8.1 ± 2.3	7.4 ± 1.7	0.0006
<i>TS</i>							
2R2R ( <i>n</i> = 22)	2R:0.50	18.3 ± 8.4	37.2 ± 26.4	<0.0001	8.2 ± 2.7	7.5 ± 2.1	0.25
2R3R ( <i>n</i> = 46)	3R:0.50	17.4 ± 5.5	40.1 ± 30.9	<0.0001	8.8 ± 4.5	8.1 ± 3.9	0.002
3R3R ( <i>n</i> = 23)		15.9 ± 5.3	42.7 ± 34.6	<0.0001	7.9 ± 1.5	7.4 ± 1.6	0.03

Data are presented as mean ± SD

\* *p* = 0.01, and \*\* *p* = 0.0006 ANOVA test. The genotype distribution for each polymorphism was tested for possible deviation from HWE

published findings (Mansoor et al. 2011). The increase, 23.0 ± 30.6 nmol/L (mean ± SD), in the concentration of serum folate and a decrease, 0.7 ± 1.4 μmol/L (mean ± SD), in the concentration of p-tHcy after folic acid supplementation, 0.8 mg/day, for 2 weeks, are similar to the findings, in healthy subjects, reported previously, increase in serum folate 18.0 ± 1.5 nmol/L (mean ± SD) and a decrease in p-tHcy 0.9 ± 0.4 μmol/L (mean ± SD) (Araki et al. 2006). It has also been demonstrated that folic acid dose, 0.8 mg/day given to healthy subjects, for 12 weeks, increased the concentration of serum folate from 12.9 ± 3.6 to 53.4 ± 28.3 nmol/L (mean ± SD) and reduced the concentration of p-tHcy from 11.5 ± 2.8 to 9.6 ± 2.2 μmol/(mean ± SD) (change about 25 %) (Van Oort et al. 2003). It has been reported that the higher is the baseline concentration of p-tHcy, the greater is the

reduction in the concentration of p-tHcy after vitamin B<sub>12</sub> or folic acid therapy (Mansoor et al. 1999; Homocysteine lowering trialists' collaboration 2005; Green 2011; Mansoor et al. 2013).

The concentration of serum folate, 5-methyl-THF, increased significantly higher in subjects with higher age than in subjects with the lower age (Table 2). This observation may suggest that age may affect folate kinetics during increased bioavailability of folate. Probably, the concentration of serum folate increases when the folate-binding sites on the intracellular proteins are saturated or the cells are unable to utilize the surplus folate in the regular biochemical reactions.

The baseline concentration of serum folate was not altered significantly by all polymorphisms in folate genes as reported previously (Table 3) (Pereira et al. 2004; Devlin

**Table 4** Concentration of serum folate associated with combined polymorphisms before and after supplementation with folic acid

			Baseline serum folate	<i>p</i> value for the comparison between polymorphism combinations	After 2 weeks serum folate	<i>p</i> value for the comparison between polymorphism combinations
<i>MTHFR-677C&gt;T</i>						
<b>MTHFR-1298A&gt;C</b>						
AA	CC	(12)	19.6 ± 7.5		36.3 ± 8.8* <sup>1</sup>	
AC+CC	CC	(28)	17.3 ± 5.6		55.6 ± 47.2	AC+CC,CC-AC+CC,CT+TT ( <i>p</i> = 0.005)
AA	CT+TT	(34)	17.2 ± 6.1		35.2 ± 21.9	
AC+CC	CT+TT	(17)	15.5 ± 6.8		29.6 ± 11.1	
<b>MTHFD1-1958G&gt;A</b>						
GG	CC	(13)	17.7 ± 6.5		50.1 ± 44.3* <sup>2</sup>	
GA+AA	CC	(27)	18.1 ± 6.2		49.3 ± 39.0	GA+AA,CC-GG,CT+TT ( <i>p</i> = 0.003)
GG	CT+TT	(20)	16.5 ± 5.8		29.1 ± 10.0	
GA+AA	CT+TT	(31)	16.7 ± 6.7		35.9 ± 22.6	
<b>MTR-2756A&gt;G</b>						
AA	CC	(24)	16.6 ± 6.2		54.3 ± 45.9* <sup>3</sup>	
AG+GG	CC	(16)	20.1 ± 5.9		42.2 ± 29.1	
AA	CT+TT	(30)	17.8 ± 6.9		35.1 ± 22.3	
AG+GG	CT+TT	(21)	15.0 ± 5.0		30.3 ± 11.7	
<b>DHFR 19del</b>						
WW	CC	(18)	16.3 ± 5.4		50.8 ± 43.5	
WD+DD	CC	(22)	19.3 ± 6.6		48.4 ± 37.9	
WW	CT+TT	(16)	16.2 ± 5.1		33.4 ± 22.5	
WD+DD	CT+TT	(35)	16.8 ± 6.8		33.2 ± 17.3	
<b>TS</b>						
3R3R	CC	(12)	16.4 ± 5.3		47.7 ± 41.0* <sup>4</sup>	
2R2R + 2R3R	CC	(28)	18.7 ± 6.6		50.4 ± 40.5	2R2R+2R3R,CC-2R2R-2R3R,CT+TT ( <i>p</i> = 0.008)
3R3R	CT+TT	(11)	15.3 ± 5.4		37.7 ± 27.6	
2R2R + 2R3R	CT+TT	(40)	17.0 ± 6.5		31.9 ± 15.7	
<b>RFC1-80G&gt;A</b>						
GG	CC	(14)	17.6 ± 6.1* <sup>1</sup>		34.8 ± 11.3* <sup>5</sup>	
GA	CC	(15)	19.2 ± 6.9		63.3 ± 44.8	GA, CC-GG,CT+TT ( <i>p</i> < 0.0001)
AA	CC	(11)	16.8 ± 5.6		48.0 ± 52.0	
GG	CT+TT	(12)	15.4 ± 3.5		26.1 ± 10.0	GA,CC-GA,CT+TT ( <i>p</i> = 0.0002)
GA	CT+TT	(26)	14.7 ± 4.7	GA,CT+TT-AA,CT+TT ( <i>p</i> = 0.002)* <sup>2</sup>	30.4 ± 17.9	
AA	CT+TT	(13)	21.7 ± 8.4		46.9 ± 22.0	

Data are presented as mean ± SD. Number of subjects in parentheses. Serum folate concentration is given in nmol/L. ANOVA test

#<sup>1</sup> *p* = 0.03, #<sup>2</sup> *p* = 0.002, \*<sup>1</sup> *p* = 0.02, \*<sup>2</sup> *p* = 0.03, \*<sup>3</sup> *p* = 0.03, \*<sup>4</sup> *p* = 0.04, \*<sup>5</sup> *p* = 0.0003

et al. 2006; Zappacosta et al. 2014; Yang et al. 2008; Anderson et al. 2013; Nelen et al. 1998; DeVos et al. 2008). After the folic acid supplementation, the concentration of serum folate was changed significantly by MTHFR-677>T polymorphisms (Table 3). However, the combination of MTHFR677 and RFC1-80 had a significant impact on the baseline concentration of serum folate (Table 4). The highest baseline serum folate concentration, 21.7 ± 8.4 nmol/L (mean ± SD), was detected in subjects

with RFC1-80AA and MTHFR-677 CT+TT combination, whereas the lowest baseline serum folate concentration, 14.7 ± 4.7 nmol/L, was measured in subjects with RFC1-80 GA and MTHFR-677CT+TT combination (*p* = 0.002) (Table 4). Furthermore, after folic acid supplementation, the subjects with MTHFR-677 CC and RFC1-80GA polymorphisms had the greatest increase in the concentration of serum folate, whereas the subjects with combination of MTHFR677 CT+TT and RFC1-80GG had the

**Table 5** Concentration of p-tHcy associated with combined genotypes before and after supplementation with folic acid

			Baseline p-tHcy	<i>p</i> value for the comparison between combined polymorphisms	2 weeks p-tHcy	<i>p</i> value for the comparison between combined polymorphisms
<i>MTHFR-677C&gt;T</i>						
<b>MTHFR-1298A&gt;C</b>						
AA	CC	(12)	6.6 ± 2.2* <sup>1</sup>	AA,CC-AA,CT+TT ( <i>p</i> = 0.0003)	6.7 ± 1.4	
AC+CC	CC	(28)	7.6 ± 1.7		7.1 ± 1.4	
AA	CT+TT	(34)	9.6 ± 4.8		8.7 ± 4.3	
AC+CC	CT+TT	(17)	8.6 ± 2.3		7.7 ± 1.8	
<b>MTHFD1-1958G&gt;A</b>						
GG	CC	(13)	7.7 ± 1.6* <sup>2</sup>	GA+AA,CC-GA+AA, CT+TT ( <i>p</i> = 0.004)	7.5 ± 1.3	GA+AA,CC-GA+AA,CT+TT ( <i>p</i> = 0.002)
GA+AA	CC	(27)	7.2 ± 2.1		6.8 ± 1.4	
GG	CT+TT	(20)	8.8 ± 1.8		7.5 ± 1.4	
GA+AA	CT+TT	(31)	9.6 ± 5.1		8.9 ± 4.5	
<b>MTR-2756A&gt;G</b>						
AA	CC	(24)	7.6 ± 2.2* <sup>3</sup>	AG+GG,CC-AG+GG,CT+TT ( <i>p</i> = 0.004)	7.3 ± 1.6	
AG+GG	CC	(16)	6.9 ± 1.2		6.5 ± 0.8	
AA	CT+TT	(30)	8.8 ± 2.2		8.0 ± 2.1	
AG+GG	CT+TT	(21)	10.0 ± 6.2		8.9 ± 5.3	
<b>DHFR 19del</b>						
WW	CC	(18)	7.8 ± 1.8* <sup>4</sup>	WD+DD,CC-WW,CT+TT ( <i>p</i> = 0.002)	7.1 ± 1.2	
WD+DD	CC	(22)	7.0 ± 2.0		6.9 ± 1.6	
WW	CT+TT	(16)	9.2 ± 2.4		8.3 ± 2.0	
WD+DD	CT+TT	(35)	9.3 ± 4.8		8.4 ± 4.3	
<b>TS</b>						
3R3R	CC	(12)	7.6 ± 1.8* <sup>5</sup>	2R2R + 2R3R,CC-2R2R + 2R3R, CT+TT ( <i>p</i> = 0.008)	7.0 ± 1.6	
2R2R + 2R3R	CC	(28)	7.2 ± 2.0		7.0 ± 1.3	
3R3R	CT+TT	(11)	8.2 ± 0.8		7.8 ± 1.5	
2R2R + 2R3R	CT+TT	(40)	9.6 ± 4.7		8.5 ± 4.1	
<b>RFC1-80G&gt;A</b>						
GG	CC	(14)	7.9 ± 2.1* <sup>6</sup>	GA,CC-GA,CT+TT ( <i>p</i> = 0.001)	7.3 ± 1.5	
GA	CC	(15)	6.9 ± 1.9		6.9 ± 1.3	
AA	CC	(11)	7.3 ± 1.6		6.7 ± 1.5	
GG	CT+TT	(12)	8.3 ± 1.7		7.5 ± 1.4	
GA	CT+TT	(26)	9.8 ± 5.4		8.6 ± 4.5	
AA	CT+TT	(13)	9.2 ± 2.8		8.6 ± 2.8	

Data are presented as mean ± SD. Number of subjects in the parentheses. The concentration of p-tHcy is given μmol/L. ANOVA test

\*<sup>1</sup> *p* = 0.002, \*<sup>2</sup> *p* = 0.007, \*<sup>3</sup> *p* = 0.007, \*<sup>4</sup> *p* = 0.006, \*<sup>5</sup> *p* = 0.006, \*<sup>6</sup> *p* = 0.01

lowest concentration of serum folate 63.3 ± 44.8 versus 26.1 ± 10.0 nmol/L (mean ± SD) (*p* < 0.0001) (Table 4). These findings suggest that MTHFR-677 CC, probably, ensures increased formation of reduced folates after folic acid supplementation and RFC1-80GA affects their

transport across the cell membrane. Therefore, combinations of RFC1-80G>A and MTHFR-677C>T polymorphisms may affect the bioavailability of intracellular and extracellular folate and as a result may disturb the folate homeostasis after folic acid supplementation in healthy



**Table 6** Concentrations of serum folate, serum vitamin B<sub>12</sub>, intake of B-vitamins and intake of protein according to the genotypes

	Serum folate (nmol/L)	Folate intake (μg/day)	Serum vitamin B <sub>12</sub> (pmol/L)	Vitamin B <sub>12</sub> intake (μg/day)	Vitamin B <sub>2</sub> intake (μg/day)	Vitamin B <sub>6</sub> intake (mg/day)	p-Hcy (μmol/L)	Protein intake (g/day)
All subjects	17.3 ± 6.0 (16.5)	286 ± 97 (282)	316 ± 134 (286)	6.8 ± 3.5 (6.2)	2.0 ± 0.9 (1.7)	1.7 ± 0.5 (1.7)	8.2 ± 3.2 (7.7)	96.1 ± 26.8 (90.5)
<i>MTHFR-677C&gt;T</i>								
CC (n = 41)	18.3 ± 5.9 (17.2)	289 ± 106 (288)	275 ± 79 (261)*	7.2 ± 4.3 (5.9)	2.0 ± 0.8 (1.8)	1.7 ± 0.5 (1.7)	7.2 ± 1.7 (7.1)*	95.6 ± 23.0 (91.8)
CT (n = 46)	16.3 ± 6.0 (15.3)	282 ± 95 (273)	324 ± 127 (311)	6.5 ± 2.7 (6.0)	2.0 ± 0.9 (1.7)	1.7 ± 0.6 (1.7)	8.5 ± 2.1 (8.3)	97.4 ± 31.0 (89.6)
TT (n = 9)	17.6 ± 6.9 (14.8)	292 ± 70 (291)	459 ± 244 (336)	7.3 ± 3.1 (6.6)	1.8 ± 0.7 (1.6)	1.7 ± 0.3 (1.5)	11.5 ± 8.7 (8.5)	90.9 ± 18.3 (87.0)
<i>MTHFR-1298A&gt;C</i>								
AA (n = 47)	17.9 ± 6.4 (16.6)	274 ± 89 (267)	329 ± 148 (318)	6.5 ± 2.8 (6.0)*	1.8 ± 0.9 (1.7)*	1.6 ± 0.5 (1.6)	8.3 ± 4.1 (7.5)	91.5 ± 24.5 (87)*
AC (n = 38)	16.7 ± 6.1 (16.1)	305 ± 105 (292)	305 ± 124 (275)	7.6 ± 4.3 (7.1)	2.2 ± 0.9 (2.1)	1.9 ± 0.6 (1.8)	8.3 ± 2.1 (8.1)	106.0 ± 29.6 (102)
CC (n = 11)	16.5 ± 4.8 (16.3)	269 ± 100 (276)	301 ± 106 (273)	5.4 ± 1.5 (5.5)	1.6 ± 0.3 (1.6)	1.6 ± 0.4 (1.5)	7.1 ± 1.6 (6.8)	81.5 ± 12.2 (82.1)
<i>RFC1-80G&gt;A</i>								
GG (n = 27)	16.3 ± 5.1 (15.2)	291 ± 114 (272)	275 ± 79 (257)	7.1 ± 5.3 (5.8)	1.9 ± 0.9 (1.7)	1.7 ± 0.6 (1.6)	7.8 ± 1.5 (8.1)	93.2 ± 29.0 (85.5)
GA (n = 44)	17.0 ± 6.0 (16.8)	278 ± 93 (284)	345 ± 148 (299)	6.5 ± 2.3 (6.1)	1.9 ± 0.7 (1.7)	1.7 ± 0.5 (1.6)	8.5 ± 4.3 (7.6)	95.9 ± 22.8 (90.4)
AA (n = 25)	18.8 ± 7.1 (16.6)	296 ± 89 (285)	310 ± 147 (282)	7.2 ± 2.9 (6.4)	2.1 ± 1.1 (1.7)	1.8 ± 0.5 (1.8)	7.9 ± 2.2 (7.6)	99.6 ± 32.0 (93.8)
<i>MTHFD1-1958G&gt;A</i>								
GG (n = 34)	16.9 ± 6.4 (16.7)	293 ± 90 (288)	329 ± 159 (299)	6.4 ± 1.8 (6.5)	2.0 ± 0.7 (1.9)	1.7 ± 0.5 (1.7)	8.2 ± 2.0 (7.7)	97.4 ± 25.7 (88.8)
GA (n = 43)	16.6 ± 4.8 (15.7)	290 ± 108 (288)	308 ± 107 (282)	7.2 ± 4.4 (6.2)	2.0 ± 1.0 (1.7)	1.7 ± 0.6 (1.7)	8.4 ± 4.3 (7.5)	97.4 ± 29.6 (95.5)
AA (n = 19)	19.5 ± 7.7 (18.3)	264 ± 88 (237)	312 ± 144 (277)	7.0 ± 3.6 (5.7)	1.8 ± 0.7 (1.7)	1.7 ± 0.5 (1.5)	7.6 ± 1.8 (7.7)	90.8 ± 22.9 (82.8)
<i>MTR-2756A&gt;G</i>								
AA (n = 57)	17.5 ± 6.3 (16.5)	287 ± 101 (267)	322 ± 145 (294)	7.0 ± 4.0 (6.2)	2.0 ± 0.9 (1.7)	1.8 ± 0.6 (1.7)	8.0 ± 1.9 (8.1)	98.5 ± 26.8 (92.4)
AG (n = 36)	17.1 ± 5.9 (16.6)	292 ± 92 (288)	304 ± 110 (278)	6.9 ± 2.5 (6.3)	2.0 ± 0.7 (1.8)	1.7 ± 0.5 (1.7)	8.4 ± 4.7 (7.3)	94.4 ± 26.8 (91.8)
GG (n = 3)	15.3 ± 3.6 (13.2)	201 ± 61 (208)	358 ± 198 (267)	6.9 ± 2.5 (6.3)	1.1 ± 0.2 (1.0)	1.1 ± 0.4 (0.9)	9.3 ± 4.1 (7.2)	68.8 ± 11.5 (73.5)
<i>DHFR-19del</i>								
WW (n = 38)	16.4 ± 5.2 (15.7)	286 ± 92 (291)	322 ± 147 (278)	5.6 ± 1.7 (5.5)*	1.8 ± 0.7 (1.7)	1.7 ± 0.6 (1.7)	8.1 ± 1.9 (8.2)	92.4 ± 25.4 (86.7)
WD (n = 37)	17.7 ± 6.4 (17.0)	288 ± 104 (284)	311 ± 130 (285)	8.2 ± 4.6 (7.1)	2.1 ± 1.0 (1.7)	1.7 ± 0.5 (1.7)	8.3 ± 4.7 (7.4)	98.1 ± 26.6 (91.3)
DD (n = 21)	18.1 ± 6.9 (16.8)	281 ± 99 (264)	314 ± 119 (329)	6.6 ± 2.5 (5.8)	1.9 ± 0.8 (1.9)	1.7 ± 0.5 (1.7)	8.0 ± 2.2 (8.1)	99.1 ± 29.9 (96.2)
<i>TS</i>								
2R2R (n = 24)	18.1 ± 7.5 (17.2)	301 ± 86 (297)	341 ± 157 (329)	8.1 ± 5.5 (6.2)	2.1 ± 0.9 (1.7)	1.8 ± 0.4 (1.7)	7.8 ± 2.5 (7.6)	102.0 ± 26.2 (95.4)
2R3R (n = 49)	17.5 ± 5.6 (16.7)	283 ± 99 (273)	316 ± 142 (273)	6.2 ± 2.1 (5.8)	1.9 ± 0.7 (1.7)	1.7 ± 0.6 (1.7)	8.6 ± 4.1 (7.6)	94.2 ± 25.9 (88.8)
3R3R (n = 24)	15.9 ± 5.6 (14.2)	277 ± 107 (271)	293 ± 86 (290)	6.9 ± 2.8 (6.9)	2.0 ± 1.0 (1.7)	1.6 ± 0.5 (1.5)	7.7 ± 1.3 (7.9)	94.0 ± 29.5 (87.6)

Data are presented as mean ± SD and median in parentheses

\* Significant difference between the groups ( $p < 0.05$ )

subjects as well as in patients with different diseases (Table 4) (Dufficy et al. 2006). These observations are novel and may suggest that polymorphism combinations, in folate metabolizing genes, may have significant impact on the concentration of serum folate in populations consuming high-dose folic acid supplements and folic acid-fortified foods on daily basis.

As shown in Table 4, MTHFR-677CC and MTHFR-1298AC+CC genotypes seem to have an opposite effect on the concentration of serum folate after folic acid supplementation, which is in accordance with a previously published report (Yang et al. 2008).

In subjects on folic acid supplementation, trial I, the concentration of serum folate was still significantly higher after the washout period and the placebo treatment than the baseline concentration. This observation suggests that it takes more than 30 days before the concentration of serum folate reaches to its baseline concentration after termination of folic acid supplements. Previously, even a 30-week washout period after a folic acid supplementation 400 µg/day for 12 weeks seemed not to be adequate (Anderson et al. 2013). Thus, these findings suggest that 5-methyl-THF formed after folic acid supplementation accumulates and remains in the serum for longer periods.

The lowest concentration of p-tHcy was detected in subjects with combined polymorphism in MTHFR-677 CC and MTHFR-1298 AA,  $6.6 \pm 2.2$  µmol/L (mean + SD), whereas the subjects with MTR-2756 AG+GG and MTHFR677 CT+TT, and RFC1 GA and MTHFR677 CT+TT polymorphisms had the highest concentrations of p-tHcy among six polymorphism combinations tested in this study ( $10.0 \pm 6.2$  and  $9.8 \pm 5.4$  µmol/L (mean ± SD) (Table 5). It has been reported previously that subjects, with MTHFR-677 TT and RFC180 GG combination, have high baseline concentrations of serum homocysteine (Devlin et al. 2006). The possible explanation is the difference in the concentration of serum folate in subjects with these three polymorphism combinations, serum folate  $19.6 \pm 7.5$ ,  $15.0 \pm 5.0$  and  $14.7 \pm 4.7$  nmol/L, respectively (mean ± SD) (Table 4). The concentrations of p-tHcy and serum folate are within the normal range in these subjects. Our observations that subjects with MTHFR-677 CC, CT and TT have significantly different concentrations of p-tHcy are in accordance with an earlier study (Table 3) (Pereira et al. 2004; Devlin et al. 2006; Zappacosta et al. 2014; Yang et al. 2008; Anderson et al. 2013; Nelen et al. 1998).

Our findings as reported in Table 5 suggest that polymorphisms in genes associated with folate metabolism affect mainly the baseline concentrations of p-tHcy in healthy subjects. It will be interesting to find out whether these polymorphisms have a significant impact on the

concentrations of p-tHcy in patients with hyperhomocysteinemia after folic acid supplementation.

We observed a stepwise, gradient, increase in the concentration of serum vitamin B<sub>12</sub> in subjects with MTHFR-677 polymorphisms, CC =  $275 \pm 79$  pmol/L, CT =  $324 \pm 127$  pmol/L and TT =  $459 \pm 244$  pmol/L (mean ± SD) (Table 6). The question is to measure the concentration of serum methylmalonic acid (MMA) and mean corpuscle volume (MCV) in TT individuals to find out whether they have lower intracellular concentrations of vitamin B<sub>12</sub> than the individuals with CC and CT genotypes. Or this is a random finding? Similarly, intake of vitamin B<sub>12</sub>, vitamin B<sub>2</sub> and proteins may be characterized according to genetic polymorphisms (Table 6); however, we do not have any evidence, which suggests that genetic polymorphisms may affect the intake of micronutrients by healthy subjects. More studies may be required to address these issues.

#### Possible clinical consequences

Probably, small serum folate concentration <10 nmol/L causes an increase in the concentration of p-tHcy, and serum folate concentration ≥12 nmol/L keeps p-tHcy concentration <15 µmol/L. Furthermore, serum folate concentration >16 nmol/L in mothers seems to reduce the risk of NTD in babies (Daly et al. 1995; Shane 2010; Etheredge et al. 2012; Us 1996; Green 2011). Thus, unphysiological serum folate concentration, ≥50 nmol/L, may not cause further reduction in the concentration of p-tHcy, neither impact one-carbon metabolism nor affect blood cell homeostasis (Table 5). Previously, elderly, who consumed 600 µg folic acid added in the bread, had serum folate concentration from 19.0 to 49.5 nmol/L and folic acid concentration from 1.03 to 7.22 nmol/L (Kelly et al. 1997). It was also reported that the individuals who had serum folate concentrations <50 nmol/L had only 2.3 % folic acid, but the individuals who had serum folate concentrations >50 nmol/L had 15.7 % folic acid in the serum (Pfeiffer et al. 2004). These observations suggest that after folic acid supplementation, folic acid and 5-methyl-THF accumulate in the blood in subjects on high-dose folic acid supplementation. Therefore, the question is whether individuals using high-dose folic acid supplements for long periods may be exposed to transient folic acid stress? Previously, it was reported that folate and zinc had a mutual inhibitory effect at the site of intestinal transport or absorption (Ghishan et al. 1986). It was also reported that women with the highest quartile folate and the lowest quartile zinc were vulnerable to fetomaternal complications (Mukherjee et al. 1984). Recently, it was demonstrated that high-dose folic acid supplements were associated with adverse effects on fetal mouse

development (Mikael et al. 2013). It was also shown that folic acid supplements (400 µg) given every other day to individuals who were consuming diet providing about 3.5 mg zinc were associated with increased loss of zinc via feces (Milne et al. 1984). In patients with cervical dysplasia, the concentration of zinc in blood cells was not reduced significantly, when they consumed 10 mg folic acid daily for 2–4 months (Butterworth et al. 1988). A recent report suggested that high-dose folic acid might promote the progression of mammary tumors in rat (Manshadi et al. 2014). Furthermore, a clinical trial based on vitamins folic acid, vitamin B<sub>12</sub> and vitamin B<sub>6</sub> supplements was terminated before time due to the concern of possible adverse effects of the B-vitamin supplements in patients with heart disease, even though the concentration of p-tHcy was reduced about 30 % (Ebbing et al. 2008). Nevertheless, no link between folic acid supplementation and CVD and cancer was established (Clarke et al. 2010).

### Limitations and strengths

The washout period in our investigation was short. However, it does not seem to affect the outcome of folic acid supplementation. The number of study subjects might be higher to explore the effect of polymorphisms and age on the concentration of serum folate. However, when the data collected from, subjects on folic acid supplementation participating in, trial I and trial II were combined ( $n = 91$ ), the effect of polymorphism combinations was significant on the pre- and post-intervention concentration of serum folate and p-tHcy. Therefore, in our view, our findings might be useful for designing new studies to assess the effects of increased folic acid consumption on trace element homeostasis and on the concentration of biomarkers for CVD and cancer.

### Conclusion

Combination of MTHFR-677 and RFC-1 polymorphisms has a profound impact on the concentration of serum folate in healthy subjects after short-term folic acid supplementation. Furthermore, polymorphisms in genes coding for folate metabolism affect the baseline concentration of p-tHcy in healthy subjects.

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**Conflict of interest** There is no conflict of interest.

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