

The MTHFR 677 C→T genotype modifies the relation of folate intake and status with plasma homocysteine in middle-aged and elderly people

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Background: A high plasma concentration of total homocysteine has been linked to a higher risk of cardiovascular disease. Subjects homozygous for the methylenetetrahydrofolate reductase (MTHFR) 677C→T mutation have depressed folate and elevated homocysteine concentrations. They may have increased folate requirements compared to subjects with CT and CC genotypes.

Objective: We investigated whether MTHFR C677T genotypes differ in their associations of 1. folate intake with folate status, and 2. folate status/folate intake with plasma homocysteine concentrations. We also investigated in these three genotypes the effect of one year folic acid supplementation (800 µg/day) on serum folate and plasma homocysteine.

Design: In a double blind randomised placebo-controlled trial, 815 volunteers aged 50-70 years (n= 312 CC, 378 CT and 125 TT) with homocysteine above 13 µmol/L at screening, were allocated to daily folic acid (800 µg) or placebo treatment during one year.

Results: At baseline, the median folate intake was 194 µg/day and did not differ between genotypes. Subjects with the TT genotype had 13% lower levels of serum folate and 8% higher homocysteine compared with CC subjects. At an intake level above 215 µg/day (upper quartile) subjects with the TT genotype had similar homocysteine compared with subjects with the CC or CT genotypes with folate intakes below 138 µg/day (bottom quartile). This indicates a higher folate requirement of the TT genotype. After one year of folic acid supplementation homocysteine decreased by 35% in the total study population. Subjects with the TT genotype had a 4.3 µmol/L (-40%) decrease of homocysteine compared to placebo, which was significantly greater (p<0.0001) than that of subjects with the CC genotype (-3.2 µmol/L, -32%) or the CT genotype (-3.0 µmol/L, -31%). After one year supplementation, mean homocysteine of subjects with the TT, CT and CC genotypes were 9.2; 9.6 and 9.7 µmol/L respectively.

Conclusions: Subjects with the TT genotype need an additional folate intake of at least 75-100 µg/day to reach similar homocysteine as those with the CC or CT genotypes. After one year of 800 µg/day folic acid supplementation, subjects with the TT genotype attained similar low-normal homocysteine as subjects with the CC or CT genotypes.

Keywords: methylenetetrahydrofolate reductase; total homocysteine; folate intake; serum folate; red blood cell folate; elderly

An elevated concentration of plasma total homocysteine has been recognized as a risk factor for vascular disease (1). Environmental (e.g. nutrition and life-style factors) and genetic factors determine homocysteine concentrations (2, 3). A common methylenetetrahydrofolate reductase (MTHFR) polymorphism, i.e. MTHFR 677C→T, has been associated with elevated homocysteine, mainly when folate status is low (4), and with 16 and 26% increased risks of coronary heart disease and stroke (5, 6). Individuals with the TT genotype have reduced MTHFR activity (5), which may result in a lower 5-methyltetrahydrofolate concentration compared with subjects with the CC genotype (7). Consequently, subjects with the MTHFR 677TT genotype have less 5-methyltetrahydrofolate available for homocysteine removal.

The MTHFR 677C→T genotype is likely to influence folate requirements. Based on cross-sectional data from a large survey in the Netherlands, subjects with the TT genotype needed at least 10% higher dietary folate than CT or CC subjects to achieve comparable plasma folate concentrations (8). Thus, subjects with the thermolabile MTHFR may have a genetic susceptibility for lower folate concentrations, higher homocysteine concentrations and higher risk of cardiovascular disease.

Can subjects with the TT genotype attain the same homocysteine concentrations as subjects with the CT and CC genotypes upon folic acid supplementation? Several trials (9-15) (table 1) have shown that subjects with MTHFR TT respond to folic acid supplementation, in a manner that the relative change of homocysteine is greater than in subjects with the CC genotypes. However, the final homocysteine concentration usually remains slightly higher in subjects with the TT genotype than in subjects with the CT or

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Table 1. Trials investigating the effect of folic acid supplementation on homocysteine per MTHFR 677C→T genotype

Study	Subjects	Treatment	Duration		MTHFR GENOTYPE		
					TT (n)	CT (n)	CC (n)
Woodside et al. (1998)	132 healthy men with homocysteine > 8.34 µmol/l	1 mg/d folic acid + 7.2 mg/d B ₆ + 0.02 mg/d B12 or placebo	8 weeks	In group treated with B-vitamins	(n=14)	(n=23)	(n=13)
				Baseline tHcy* (µmol/l)	11.2	10.3	9.1
				After 8 weeks	8.2	6.9	6.6
				Change % (vs placebo)	-26	-33	-27
Ashfield-Watt et al. (2002)	126 healthy subjects (age: 18-65)	0.4 mg/d folic acid or 0.2 mg/d extra dietary folate or	3 x 4 months (cross-over)		(n=42)	(n=42)	(n=42)
				Baseline tHcy (µmol/l)	12.5	9.3	8.8
				Supplement 4 weeks	9.5	8.1	8.0
				Change%	-24	-13	-9
				Diet 4 weeks	10.2	8.2	7.9
				Change%	-18	-12	-10
Fohr et al. (2002)	160 healthy women (age: 19-39)	0.4 mg/d folic acid, or a placebo	8 weeks		(n=5)	(n=25)	(n=21)
				Baseline tHcy (µmol/l)	7.4	8.5	8.8
				After 8 weeks	6.8	7.0	7.7
				Change % (vs placebo)	-7.9	-19.4	-14.6
Guinotte et al. (2003)	43 healthy women (age: 18-43)	Depletion: 7 weeks low folate diet; 135 µg/d DFE. Repletion: 0.4 mg/d or 0.8 mg/d for 7 weeks	14 weeks		(n=17)	(n=12)	(n=14)
				Depletion Baseline tHcy	5.4	5.9	5.3
				Week 7	7.3	7.6	6.7
				Change %	27.1	22.4	20.9
				400 µg/d DFE	(n=9)	(n=6)	(n=7)
				Baseline tHcy (µmol/l)	7.5	7.2	6.3
				After 14 weeks	5.8	5.5	5.1
				Change %	-22.7	-31.0	-23.5
				800 µg/d DFE	(n=8)	(n=6)	(n=7)
				Baseline tHcy (µmol/l)	7.0	7.2	8.2
				After 14 weeks	5.1	5.5	6.1
				Change %	-37.3	-30.9	-34.4
Kauwell et al. (2003)	41 healthy women (age: 20-30 years)	Depletion: 7 weeks low folate diet; 115 µg/d DFE** (1.7x supplementary folic acid); Repletion: 0.4 mg/d for 7 weeks	14 weeks		(n=19)	(n=0)	(n=22)
				Baseline tHcy (µmol/l)	7.1		6.6
				Week 7	10.4		9.0
				Change %	-31.7		-30.3
				Week 14	8.6		7.5
				Change %	-17.4		-16.1
Hiraoka et al. (2004)	100 young healthy women (age: 19-29 years)	4 weeks 200 µg/day folic acid supplements followed by a 4-week wash out period and then 4 weeks 400 µg/day folic acid supplementation	12 weeks		(n=17)	(n=47)	(n=36)
				Baseline tHcy (µmol/l)	10.9 ^{A, B}	8.9 ^A	8.8 ^B
				After week 4 tHcy (µmol/l)	NR	NR	NR
				After week 12 tHcy (mmol/L)	B		B
Chin-San Liu et al. (2004)	23 patients with cardiovascular disease (age: 44-88)	5 mg folic acid	8 weeks		(n=4)	(n=12)	(n=7)
				Baseline tHcy (µmol/l)	22.1	13.2	13.4
				After 8 weeks	12.6	10.1	10.8
				Change %	-40	-23	-10

tHcy*: Serum total homocysteine concentrations; DFE**: The dietary folate equivalent of 1.7 is used in US as a multiplication factor for supplementary folic acid; ^{A, B} MTHFR genotypes with same superscripts significantly differ; p<0.05; NR: Not reported.

CC genotypes. As trials often contain small numbers of subjects with the TT genotype or have short supplementation periods, it is still uncertain whether subjects with the TT genotype can attain the same homocysteine concentration as those with the CT and CC genotypes upon folic acid supplementation. In this study we investigated whether the MTHFR C677T genotypes differ in their associations of 1. folate intake with folate status, and 2. folate status/folate intake with plasma homocysteine concentrations. We also investigated the effect of 1-year folic acid supplementation (800 µg/day) on serum folate and homocysteine concentrations in these three genotypes.

Subjects and methods

Subjects

Subjects were participants of an ongoing intervention study called FACIT (acronym for Folic Acid and Carotid Intima-media Thickness) that investigates whether folic acid supplementation can halt the progression of atherosclerosis and thereby decrease the risk of vascular disease. Regional blood banks and city councils in the area of Wageningen University provided 40,500 addresses of persons aged 50-70 years. These subjects were eligible for inclusion if they fulfilled the following criteria: 1) between 50 and 70 years at study entry; 2) women should have had their last menstruation longer than 2 years ago and if the uterus was removed they had to be above 55 years of age; 3) no thyroid or renal disease; 4) no use of medication that affects homocysteine concentrations (e.g. folate analogue therapy) and no use of medication that might significantly affect atherosclerosis progression (e.g. lipid-lowering drugs, hormone replacement therapy); 5) no use of supplements containing folic acid, vitamin B₆ or vitamin B₁₂; 6) plasma total homocysteine concentrations had to be between 13 and 26 µmol/L; serum vitamin B₁₂ had to be ≥200 pmol/L, and 7) compliance during the six-week run-in period had to be ≥80%. Among the 819 subjects, the genotypes of 4 subjects could not be determined, giving rise to the availability of complete data of 815 subjects for the cross-sectional analysis. After 1-year intervention complete data from analyses were available for 802 subjects, since blood indices of 13 were missing for reason of drop-out. The Medical Ethical Committee for health research of the Department of Human Nutrition of the Wageningen University approved the study and written consent was obtained from all subjects.

Study design

The FACIT study was designed as a double blind randomised controlled trial from which we used data obtained at baseline and after 12 months of intervention for the present evaluation. Eight hundred and nineteen subjects were randomised to a placebo group or a folic acid treatment group, using a 6 by 4 permuted block design. Capsules were made for this intervention by Swiss Caps AG in Switzerland. Capsules of the folic acid group were manufactured to

contain 800 µg folic acid. The folic acid content of the folic acid capsules (n=30) was measured at the beginning and at the end of the 1 year intervention period. Mean folic acid content was 0.76 mg with a range of 0.72-0.79 at the beginning and 0.69 mg folic acid (range 0.68-0.73) after one year. The placebo capsules looked similar to the folic acid capsules, both on the inside and outside. To check compliance, the subjects returned remaining capsules and a date-form of capsules that were not taken, every twelve weeks.

General measurements

Blood was collected after an overnight fast, both at baseline and after one year. Weight (kg) was determined with an electronic weight scale to the nearest 0.1 kg to calculate body mass index (kg/m²). Height (m) was measured to the nearest 0.1 cm at baseline. Systolic and diastolic blood pressures were measured eight times, in two sessions of 40 min, using a DINAMAP® Compact PRO 100 (Critikon, Tampa USA). Mean values were used for further analyses. Information on self-reported medical history, current drug use, family history of premature vascular disease, and smoking was obtained through a questionnaire. A trained research assistant reviewed the questionnaire and uncertainties, if any, were discussed with the participant.

Dietary intake

Subjects received a food frequency questionnaire on the first measurement day. The questionnaire was filled out at home and reviewed with a research assistant on the second measurement day. The food frequency questionnaire was developed by TNO, Nutrition and Food Research (16) to assess the habitual mean daily folate intake on population level in the past three months. The selection of major dietary sources of folate was made on the basis of the results of the Dutch Nutrition Survey for the sub-population of men and women of 50-70 years (n=1,278) (17). Folic-acid calculations were based on *McCance and Widdowson's Composition's of Foods* (18). Product-portion sizes were mainly based on the 'Measure and Weight' report (19). Folate intakes were estimated from the dietary data using SAS-Vovris software (20-24). All study data were entered twice and checked for discrepancies by two independent dietitians. Collection, entry and analysis of the data were done without awareness of treatment allocation.

Laboratory methods

At baseline, fasting venous blood samples were drawn for measurements of plasma homocysteine, serum folate, red blood-cell folate, serum vitamin B₁₂, serum creatinine, and serum total HDL- and LDL-cholesterol and triglycerides. These indices were also measured after one year (except for red blood cell folate, creatinine and cholesterol). EDTA-containing tubes were used for analyses of homocysteine and red blood cell folate. Samples were immediately put on melting ice and stored at -80°C

until further analysis. Homocysteine, including its protein- and non-protein-bound fractions, was measured by high performance liquid chromatography (HPLC) with fluorescence detection at the Division of Human Nutrition, Wageningen University, The Netherlands; essentially according to Ubbink and Ueland (25,26). Vitamin B₆ was measured by HPLC (27), serum folate and vitamin B₁₂ were measured with a commercial chemiluminescent immunoassay analyser (Immulite® 2000, Diagnostic Products Company, Los Angeles, USA). Red blood cell folate was measured in the same way, after haemolysis. The Hitachi 747® was used to determine plasma creatinine and lipids. DNA was extracted from frozen peripheral blood lymphocytes with a salting-out procedure. The presence of the MTHFR 677C→T genotype was assessed in the Wageningen University, using a polymerase chain reaction followed by restriction enzyme analysis with *HinFI*, according to Frosst et al. (28). Intra- and interassay coefficients of variation were <15% for all laboratory analyses.

Statistical analyses

Objective I: To investigate whether MTHFR C677T genotypes differed in their associations of 1. folate intake with folate status, and 2. folate status/folate intake with plasma homocysteine concentrations. Logarithmic transformations were used in statistical analysis because the concentrations of serum folate and red blood cell folate, dietary folate intake and plasma homocysteine proved non-Gaussian distributed. Regression analysis was done to assess the association of folate intake (independent variable) with serum folate and red blood cell folate and homocysteine (dependent variable) and the association of folate and folate status parameters (independent vari-

ables) with total homocysteine (dependent variable). When we examined the relation between folate intake and folate status we adjusted for age, sex, smoking (pack-year), serum vitamin B₁₂ and plasma vitamin B₆. In the regression models that examined the association of folate intake and folate status with homocysteine we included age, sex, smoking, serum vitamin B₁₂, plasma vitamin B₆ and alcohol consumption (g/day) as possible confounders. The choice of these confounders was based on previous literature data (3,29,30). To check whether the slopes of the associations differed between genotypes, we calculated the 95% confidence interval (CI) of the difference between the slopes of two adjusted regression lines. The slopes did not differ when 0 belonged to the CI (31). Generalized linear model (GLM) univariate analysis followed by the Tukey test was used to test differences between genotypes within quartiles and for differences within genotypes between quartiles of dietary folate intake, serum folate or red blood cell folate.

Objective II: To investigate whether the effect of 1-year folic acid supplementation (800 µg/day) on serum folate and homocysteine concentrations differed between genotypes. For each individual, the value of baseline values was subtracted from the value at year 1. The following calculation was used to assess the changes of folate and homocysteine concentrations in the folic acid group compared to the placebo group: mean (year 1 folic acid - baseline folic acid) - mean (year 1 placebo - baseline placebo). Treatment effects within each genotype are expressed as percentage change relative to baseline values. GLM univariate analysis was used to test whether genotype has an effect on changes of serum folate or plasma homocysteine concentrations. To test whether

Table 2. Characteristics of the study population (n=815), stratified by MTHFR 677C→T genotype

	All participants	CC (n=312)	CT (n=378)	TT (n=125)
Age, y ^a	60 (5.6)	60 (5.7)	60 (5.6)	60 (5.4)
Male/Female, n	582/233	230/82	269/109	83/42
Dietary folate intake, µg/d ^b dd	194 (159-239)	197(160-241)	186(155-242)	200(168-228)
Homocysteine, µmol/l ^a c	14.4 (2.3)	14.1 (1.9)	14.2 (2.3)	15.3 (3.0) ^d
Serum folate, nmol/l ^a	12.5 (4.5)	13.1 (4.7) ^d	12.3 (4.2)	11.4 (4.4)
Red blood cell folate, nmol/l ^a	696 (267)	715 (258)	661 (236) ^d	750 (348)
Serum B ₆ , nmol/l ^b	31 (24-41)	32 (24-43)	32 (23-41)	31(24-39)
Serum B ₁₂ , pmol/l ^b	300 (252-383)	284 (239-353)	292 (245-370)	293 (240-370)
Creatinine, µmol/l ^a	93 (12)	93 (12)	92 (12)	92 (13)
Total cholesterol, mmol/l ^a	5.82 (1.1)	5.87 (1.2)	5.75 (1.0)	5.93 (1.2)
% Hypercholesterolemia ^a e g	36 (0.5)	37 (0.5)	33 (0.5)	42 (0.5)
% Hypertension ^a e h	22 (0.4)	24 (0.4)	22 (0.4)	20 (0.4)
Body mass index, kg/m ² a	27 (3.6)	27 (3.5)	26 (3.4)	27 (4.4)
Alcohol consumption, g/d ^b	12.5 (4.3- 23.5)	13.7 (4.4-23.5)	11.6 (3.6-24.1)	11.6 (5.5-20.0)
% Current smokers ^e f	166 (20)	60 (90)	82 (22)	24 (19)
% Vascular disease ^e f	97 (12)	35 (11)	46 (12)	16 (13)
% Diabetes Mellitus ^e f	26 (3)	8 (3)	13 (3)	5 (4)

^a Data are presented as the mean (SD); ^b Data are presented as the median (25-75 percentiles); ^c Mean of screening and baseline total homocysteine was used as homocysteine; ^d Significantly different from other 2 genotypes, p< 0.05 (ANOVA); ^e No significant differences between genotypes, p< 0.05 (χ²-test); ^f Data are presented as number (%); ^g Hypercholesterolaemia: total cholesterol >6.5 mmol/L, high-density lipoprotein cholesterol (LDL) < 0.9 mmol/L or use of lipid-lowering medication; ^h Hypertension: systolic blood pressure ≥160 mm Hg, diastolic blood pressure ≥95 mm or use of antihypertensive medication.

the three genotypes differed significantly for changes in serum folate and plasma homocysteine, 95% CI for the difference between means of year 0 and year 1 per genotype was calculated (32). Differences were considered significant at the 5% level (2-tailed). The values are expressed as means±standard deviation. SPSS 11.0 for Windows was used for the statistical analysis.

Results

Study population

The study group comprised 582 men and 233 women with a mean age of 60 years at baseline. The characteristics of the study population, stratified by MTHFR genotype, are shown in table 2. The MTHFR C677T allelic distribution did not differ significantly from the calculated expected distribution, assuming Hardy-Weinberg equilibrium. Fifteen percent of the participants had the TT genotype for the MTHFR 677C→T mutation, 47% had the CT genotype and 38% had the CC genotype. Mean dietary folate intake did not differ between genotypes. However, serum folate was significantly lower in subjects with the TT genotype ($p=0.001$) compared to subjects with the CC genotype, with the CT subjects having intermediate serum folate (table 2). Red blood cell folate was lowest in subjects with the CT genotype and differed significantly from that of subjects with the CC ($p=0.01$) and the TT ($p=0.01$) genotypes. Subjects with the TT genotype had significantly higher homocysteine than subjects with the CT or the CC genotypes ($p<0.05$).

Cross-sectional study

Relation between dietary folate and folate status per MTHFR genotype

There was a direct association between folate intake and folate status, which is shown per MTHFR genotype in figures 1.1 and 1.2. Strikingly, at any quartile of dietary folate intake TT subjects had a higher red blood cell folate than subjects with the CC or the CT genotypes ($p<0.05$), except for the second quartile. Conversely, at any quartile of dietary folate intake, subjects with the TT genotype had lower serum folate than the CC and CT genotypes ($p<0.05$).

The strength of the association of dietary folate with serum folate did not differ between genotypes (table 3). The association of folate intake with red blood cell folate was stronger in subjects with the TT genotype than in subjects with the CC or the CT genotypes (table 4).

Relation between folate status and homocysteine per MTHFR genotype

Serum folate and red blood cell folate were inversely related with homocysteine for all genotypes, in both the crude- and adjusted analyses (table 3). For both serum and red blood cell folate, a stronger inverse association with homocysteine was found for subjects with the TT genotype, compared with those with the CC or CT genotypes (table 4).

As shown in figure 2.1, at each quartile of serum folate, subjects with the TT genotype appeared to have higher homocysteine compared with subjects with the CC or the CT genotypes. A significant difference between the TT genotype and the other two

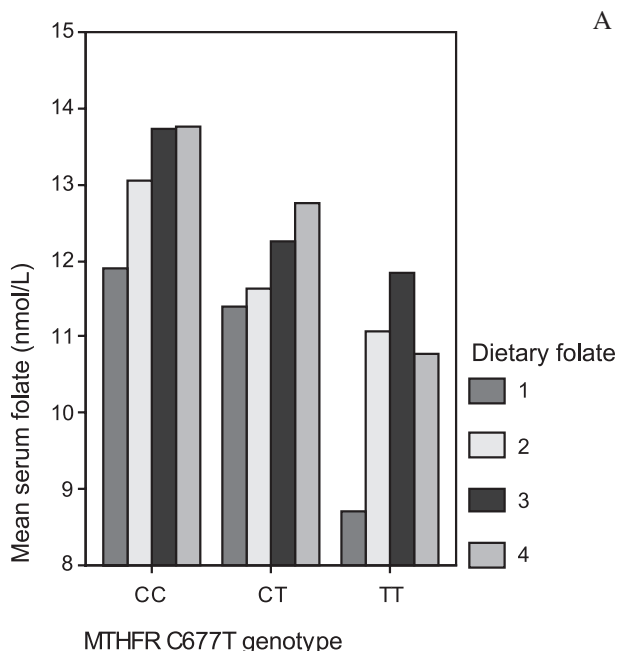


Figure 1A. Mean serum folate concentrations according to MTHFR genotypes across quartiles of dietary folate, adjusted for: age, sex, smoking, serum vitamin B₁₂ and serum vitamin B₆. Cut-off thresholds for dietary folate quartiles (1 to 4) were ≤ 137, 138 to 176, 177 to 214 and 215 to 276 µg/d.

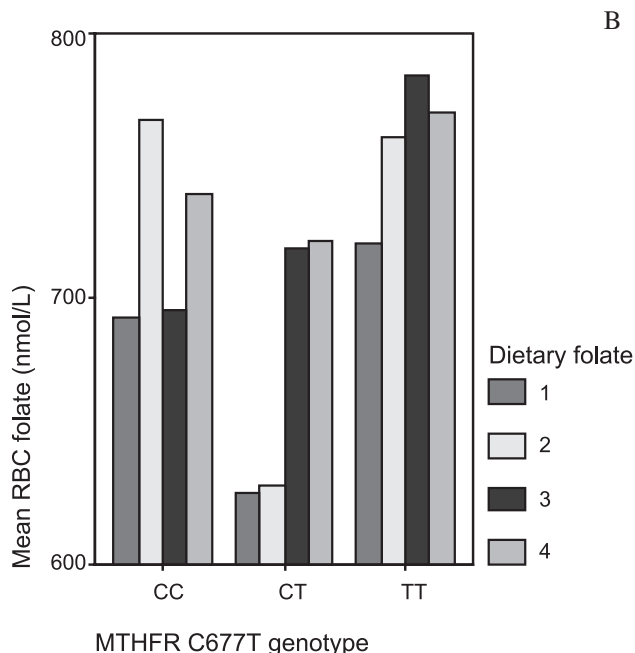


Figure 1B. Mean red blood cell (RBC) folate concentrations according to MTHFR genotype across quartiles of dietary folate adjusted for age, sex, smoking, serum vitamin B₁₂ and serum vitamin B₆ quartiles (1 to 4) were ≤ 137, 138 to 176, 177 to 214 and 215 to 276 µg/d.

genotypes was only found for the second quartile ($p < 0.05$). At red blood cell folate concentrations below the median (< 575 nmol/l, i.e. the lowest two quartiles), subjects with the TT genotype had higher homocysteine than subjects with the CC and CT genotypes (figure 2.2) ($p < 0.02$). No significant difference(s) in homocysteine between genotypes were found among subjects with red blood cell folate above the median. Thus, at low folate levels MTHFR genotype is a more important determinant of homocysteine than at high folate levels.

Relation between dietary folate and homocysteine per MTHFR genotype

Figure 3 illustrates that at any quartile of folate intake, subjects with the TT genotype had higher homocysteine than subjects with the CC or the CT genotypes. It was striking that at intake levels above 215 $\mu\text{g/day}$ (upper quartile), subjects with the TT genotype had similar homocysteine as CC or CT subjects with folate intakes below 138 $\mu\text{g/day}$ (bottom quartile). However, significant differences between subjects with the TT

genotype and subjects with the CC or CT genotypes were only found when folate intake was below the median (< 177 $\mu\text{g/day}$, i.e. the lowest two quartiles) ($p < 0.047$). Although the steepest inverse slope was seen in subjects with the TT genotype, no significant difference between genotypes was found for the strength of the association of dietary folate intake with homocysteine (tables 3 and 4). In summary, having low dietary folate renders the effect of MTHFR genotype on homocysteine more pronounced.

Intervention study

The mean concentrations of homocysteine and serum folate for the three genotypes at year 0 (baseline) and after 1 year of intervention with folic acid or placebo are depicted in table 5. The same pattern for the means of the serum folate and plasma homocysteine across genotypes as seen in the entire study population was observed in the placebo group and in the folic acid group, i.e. higher homocysteine and lower serum folate in TT subjects compared with the other two genotypes.

Table 3. Unstandardised β 's (SE) for the relation of dietary folate intake with folate status and of the latter variables with homocysteine, crude or adjusted for various confounders^a, stratified per MTHFR C677T genotype

	CC (N=312)		CT (N=378)		TT (N=125)	
Folate intake -> Red blood cell folate						
Crude	0.186	(0.230)	0.541 ^b	(0.139)	0.517	(0.408)
Model 1	0.112	(0.235)	0.537 ^b	(0.140)	0.613	(0.401)
Model 2	0.051	(0.230)	0.542	(0.144)	0.598	(0.420)
Folate intake -> Serum folate						
Crude	0.011 ^b	(0.004)	0.007 ^b	(0.002)	0.008	(0.008)
Model 1	0.013 ^b	(0.004)	0.007 ^b	(0.002)	0.008	(0.005)
Model 2	0.011 ^b	(0.004)	0.007 ^b	(0.002)	0.011 ^b	(0.005)
Red blood cell folate -> Homocysteine						
Crude	-0.001	(0.000)	-0.002	(0.000)	-0.003 ^b	(0.001)
Model 1	-0.001 ^b	(0.002)	-0.000 ^b	(0.001)	-0.007 ^b	(0.004)
Model 3	-0.001 ^b	(0.000)	-0.002 ^b	(0.000)	-0.003 ^b	(0.000)
Serum folate -> Homocysteine						
Crude	0.095 ^b	(0.022)	-0.152 ^b	(0.027)	-0.204 ^b	(0.059)
Model 1	-0.097 ^b	(0.022)	-0.151 ^b	(0.028)	-0.196 ^b	(0.050)
Model 3	-0.091 ^b	(0.023)	-0.155 ^b	(0.028)	-0.192 ^b	(0.060)
Folate intake -> Homocysteine						
Crude	-0.001	(0.002)	-0.000	(0.001)	-0.007 ^b	(0.004)
Model 1	-0.002	(0.002)	-0.001 ^b	(0.001)	-0.006	(0.003)
Model 3	-0.001	(0.001)	0.000	(0.001)	-0.010	(0.004)

^a Model 1: Adjusted for age and sex; ^a Model 2: Adjusted for: age, sex, smoking (smoking pack years), serum vitamin B₁₂ and serum vitamin B₆; ^a Model 3: Adjusted for: age, sex, smoking (smoking pack years), serum vitamin B₁₂, serum vitamin B₆ and alcohol consumption; ^b Significantly different from the other two genotypes, $P = 0.05$ (Tukey)

Table 4. The difference and 95% confidence interval between the slopes of the regression lines (unstandardised β) of MTHFR 677TT versus CC or TT versus CT subjects

	* $\beta_{\text{CC}-\beta_{\text{TT}}$	95% Ci of CT AND TT	* $\beta_{\text{CT}-\beta_{\text{TT}}$	95% CI OF CC and TT
Folate intake -> Red blood cell folate	0.105	-0.145 to 0.355	-0.056	-0.288 to 0.268
Folate intake -> Serum folate	0.150	-0.176 to 2.285	0.010	-0.267 to 0.287
Red blood cell folate -> Homocysteine	0.002	-0.236 to 0.252	0.002	-0.246 to 0.250
Serum folate -> Homocysteine	0.101	-0.115 to 0.105	-0.057	-0.307 to 0.194
Folate intake -> Homocysteine	0.009	-0.247 to 0.246	0.010	-0.236 to 0.237

* β = unstandardised β

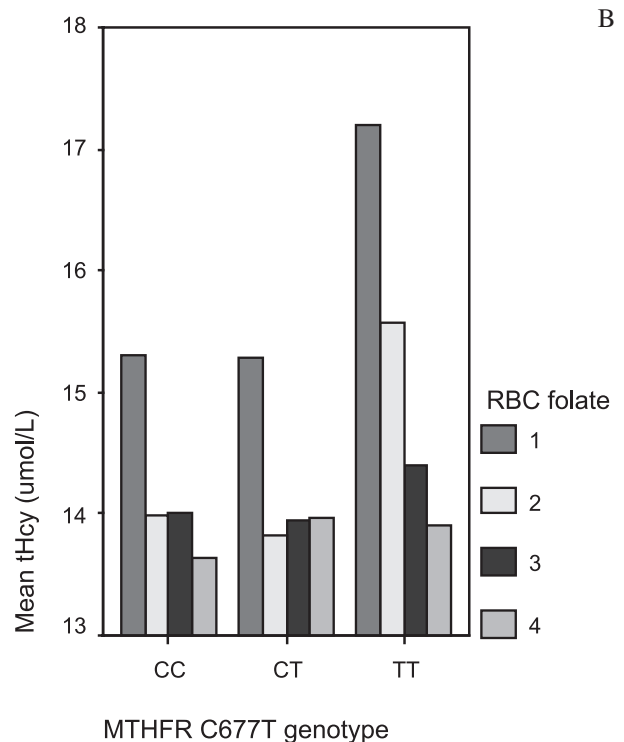
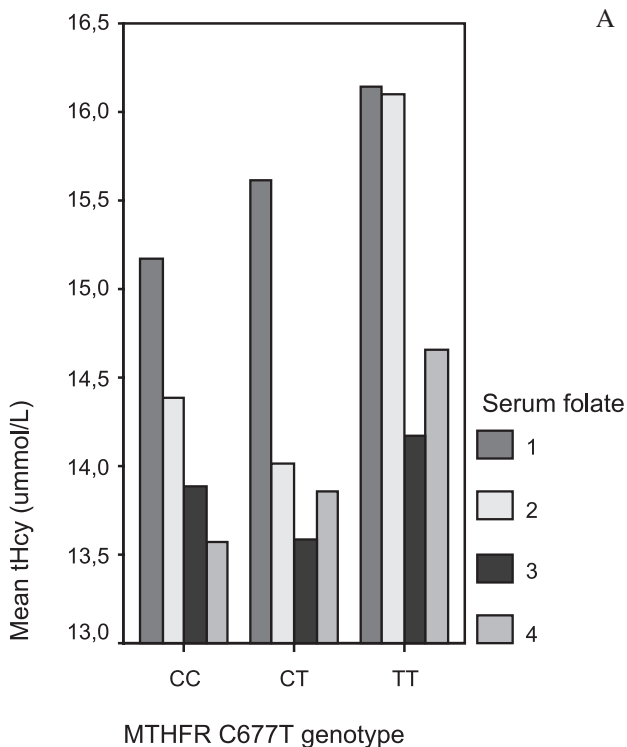


Figure 2A. Mean homocysteine (tHcy) concentrations according to MTHFR genotypes across quartiles of serum folate, adjusted for: age, sex, smoking, serum vitamin B₁₂, serum vitamin B₆ and alcohol consumption. Cut-off thresholds for serum folate quartiles (1 to 4) were ≤ 8.1, 8.2 to 10.6, 10.7 to 13.1 and 13.2 to 17.2 nmol/l.

Figure 2B. Mean homocysteine (tHcy) concentrations according to MTHFR genotypes across quartiles of serum red blood cell folate levels, adjusted for: age, sex, smoking, serum vitamin B₁₂, serum vitamin B₆ and alcohol consumption. Cut-off thresholds for serum red blood cell folate quartiles (1 to 4) were ≤ 427, 428 to 575, 576 to 734 and 735 to 998 nmol/l.

After one year, the group that took the folic acid supplement had a higher serum folate (mean: 68±35 versus 13±11 nmol/l) ($p < 0.001$) and a lower homocysteine (10±2 versus 13±3 μmol/L) ($p < 0.001$) than the placebo group ($p < 0.05$). Folate supplementation augmented serum folate by 526% among CC subjects, 596% among CT subjects and 506% among subjects with the TT genotype, compared with changes in the placebo group. The decrease in homocysteine, but not the increase in serum folate was significantly larger in the TT genotype than in the other two genotypes (table 6). In response to folic acid supplementation, homocysteine decreased by 40, 31 and 32%, compared with the placebo groups in subjects with the TT, CT and CC genotypes, respectively. At the end of year 1, mean homocysteine of subjects with the TT, CT and CC genotype were 9.2, 9.6 and 9.7 μmol/l, respectively ($p > 0.05$), as shown in table 5.

Discussion

We examined whether MTHFR C677T genotypes differ in their associations of 1. folate intake with folate status, and 2. folate status/folate intake with plasma homocysteine concentrations. Furthermore, we investigated the effect of 1-year folic acid supplementation (800 μg/day) on homocysteine-lowering within groups of the three genotypes. We found that subjects with the TT genotype had 8% higher homocysteine than subjects with the CC genotype and 7% higher homocysteine than subjects with the CT genotype. The effect of genotype on homocysteine was

especially pronounced among subjects with folate intakes below the median level of the population. Strikingly, even at the highest quartile of folate intake, subjects with TT genotype had similar homocysteine compared with subjects with CC or CT genotypes with folate intakes in the lowest quartile of folate intake. In this study the elderly population had a median folate intake of 194 μg/day, which is lower than the current 300 μg/day recommended daily allowance (RDA) in the Netherlands (33). After one year of 800 μg/day folic acid supplementation, homocysteine concentrations of all three genotypes were reduced to similar low-normal concentrations.

Cross-sectional study

The difference between homocysteine levels of MTHFR C677T TT and CC genotypes was quite modest (~8%), compared to other studies that usually reported differences of about 15-25% (5,34). This is likely to be due to the selection of subjects with high baseline homocysteine concentration (>13 μmol/L). Like many others (35,36), we observed that folate status determines the strength of the association between MTHFR C677T genotype and homocysteine: the association is stronger at low folate status. We used a food frequency questionnaire to assess folate intake, which, unlike measures of folate status, is likely to be independent of MTHFR C677T genotype. Therefore, it is much more appropriate to study the association of genotype with homocysteine by subgroups of folate intake rather than subgroups of

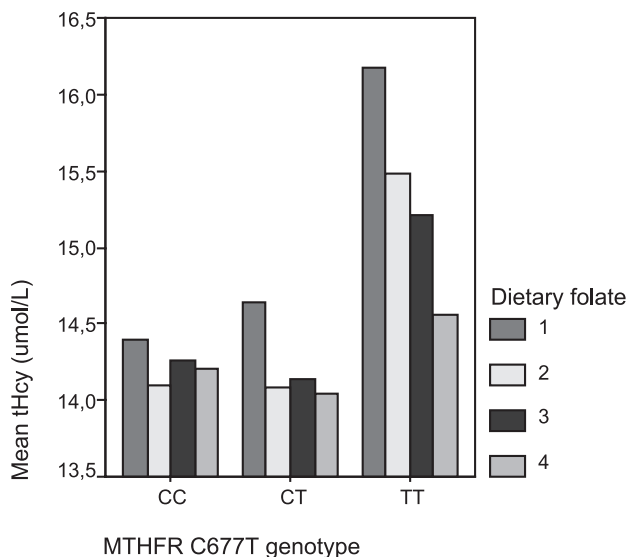


Figure 3. Homocysteine levels according to MTHFR genotypes across quartiles of dietary folate, adjusted for: age, sex, smoking, serum vitamin B₁₂, serum vitamin B₆ and alcohol consumption. Cut-off thresholds for dietary folate quartiles (1 to 4) were ≤137, 138 to 177, 178 to 214 and 215 to 276 µg/d.

folate status. Like for folate status, there was a stronger association of genotype with homocysteine when dietary folate levels are low. This observation is in line with recent findings of Hiraoka et al. (14) who studied 100 healthy women.

Contrary to what we anticipated based on previous *in vitro* reports (5, 6), subjects with the TT genotype had higher levels of red blood cell folate than subjects with the CC or CT genotypes. An explanation for this unexpected observation could be the difference in folate derivative composition between the genotypes and the different sensitivities of their chemical detec-

tion with use of the present assay. TT homozygosity has been associated with a shift in folate derivative distribution, as derived from the higher levels of non-methylated folate derivatives and the lower levels of methylated folate derivatives in red blood cells (37). Our chemical analysis measures 5-methyltetrahydrofolate, 10-formyltetrahydrofolate and 5,10-methenyltetrahydrofolate as total red blood cell folate, whereas serum mainly contains 5-methylenetetrahydrofolate. This difference might explain the observation that the direct association of dietary folate with red blood cell folate, but not with serum folate, was significantly stronger in subjects with the TT genotype, compared to subjects with the CC or CT genotypes. Perhaps a higher sensitivity to detect non-methylated folates (38) could be another explanation for the higher red blood cell folate in subjects with the TT genotype than in subjects with the CC and CT genotypes. No significant differences in white blood cell concentrations were found between genotypes, which might also explain the encountered differences ($p=0.501$). Finally, we consider our measurements of red blood cell folate to be reliable, since the analysis was done in duplicate and red blood cell folate showed the usual inverse relationship with homocysteine.

Intervention study

Several studies in the general population, have suggested that the homocysteine-lowering effect of folic acid reaches a plateau at an intake of ≈400 µg/day with higher doses of folic acid producing little additional benefit. Data on dose-finding studies that investigate such relations in each of the MTHFR C677T genotypes are, however, limited (9-14). In one of the dose-finding studies (39), subjects were randomised to placebo, 50, 100, 200, 400, 600 or 800 µg folic acid/day during twelve weeks. The authors con-

Table 5. Mean serum folate and homocysteine concentrations for the MTHFR 677C→T genotypes at year 0 (baseline) and after 1 year of intervention*

Genotype	Placebo			Supplement		
	CC	CT	TT	CC	CT	TT
Year 0 (n=815)	(n=168)	(n=191)	(n=52)	(n=144)	(n=187)	(n=73)
Serum folate (nmol/L)	13.11±4.89	12.66±4.25	10.81±4.54	13.15 ± 4.41	11.91±4.13	11.79 ± 4.34
Homocysteine (µmol/L)	14.17±1.8	14.19±2.6	15.43±3.5	14.10±1.9	14.25±2.0	15.26±2.7
Year 1 (n=802)	(n=166)	(n=189)	(n=50)	(n=141)	(n=183)	(n=73)
Serum folate (nmol/L)	13.43±1.0	13.77±11.2	12.75±3.6**	69.21±30.1	70.96±40.0	59.69±31.4
Homocysteine(µmol/L)	13.00±2.8	12.75±3.6	15.33±3.0**	9.73±2.1	9.60±1.8	9.21±1.9

*Data are presented as the mean ±SD ** Significantly different from the other two genotypes, $P<0.05$ (ANOVA).

Table 6. Change in concentration compared to placebo¹ from baseline in serum folate and homocysteine at the end of the study with respect to MTHFR 677C→T genotypes²

	Genotype		
	CC	CT	TT
Serum folate (nmol/L)	55.8 (50.92 to 60.22)	57.8 (51.40 to 62.05)	47.6 (38.16 to 55.72)
Homocysteine(µmol/L)	-3.2 (-3.68 to -2.86)	-3.1 (-3.51 to -2.79)	-4.3 (-5.26 to -3.28) ^a

¹ Change in concentration= (intervention supplementation – baseline supplementation) – (intervention placebo - baseline placebo)

² Data are presented as the mean and (95% CI of mean difference)

^a Significantly different from the other two genotypes, $P<0.05$ (GLM)

cluded that, in subjects with MTHFR TT, doses lower than 600-800 µg given for twelve weeks are too low to reach a final homocysteine that is comparable with that of subjects with the CC or CT genotypes. The minimal required folic acid dose appeared to be 800 µg/day (40). In our study, after 800 µg/day folic acid supplementation for one year the final homocysteine of subjects with the TT genotype was at least as low as for subjects with the CC and the CT genotypes. Other studies (9-15) among healthy subjects showed lower absolute changes in homocysteine than our study. Shorter duration, different treatment regimens and different inclusion criteria of those studies compared with our study may explain our high absolute changes in homocysteine. For example, we applied a >13 µmol/L homocysteine inclusion criterion at screening, whereas many others used lower concentrations as inclusion criterion (9) or did not screen for homocysteine concentration.

We conclude that subjects with the TT genotype may have increased folate requirements, since they need an additional folate intakes of at least 75-100 µg/day to reach similar homocysteine concentrations as those with the CC or CT genotypes. With long term 800 µg/day folic acid supplementation, the homocysteine of all genotypes can be reduced to similar low-normal concentrations. However, a dietary folate intake of 800 µg/day can not be achieved by the consumption of natural foods. More long-term studies need to be conducted to determine the influence of lower folic acid doses, or increased dietary folate intake, on homocysteine concentrations in the different genotypes. Based on observational studies, the 35% homocysteine decrease of our study, would translate to 15% lower ischaemic heart disease and 27% lower stroke risks (1). It should, however, be kept in mind that the clinical relevance of homocysteine reduction is uncertain, since the causal role of homocysteine in the aetiology of vascular disease is as yet unproven.

Acknowledgements and funding

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Samenvatting

Het MTHFR 677C→T-genotype verandert de relatie tussen foliumzuuriname en hoeveelheid plasmahomocysteïne bij mensen op middelbare en oudere leeftijd. Öztürk H, Durga J, van de Rest O en Verhoef P. Ned Tijdschr Klin Chem Labgeneesk 2005; 30: 208-217

Achtergrond: Een hoge plasmaconcentratie van totaal homocysteïne wordt in verband gebracht met een hoger risico op hart- en vaatziekten. Personen die homozygoot zijn voor de methylenetetrahydrofolaatreductase(MTHFR)-677C→T-mutatie hebben verlaagde folaat- en verhoogde homocysteïneconcentraties. Personen met het TT-genotype hebben mogelijk een verhoogde folaatbehoefte ten opzichte van personen met CT- en CC-genotypes.

Doel: Wij onderzochten of de MTHFR-C677T-genotypen onderling verschillen in hun associaties tussen 1. folaatname en folaatstatus, en 2. folaatstatus/folaatname en plasmahomocysteïneconcentraties. Ook onderzochten wij in deze drie genotypen de invloed van één jaar foliumzuur supplementie (800 µg/day) op het serumfolaat en het plasmahomocysteïne.

Opzet: In een dubbelblinde gerandomiseerde en placebocontroleerde studie werden 815 vrijwilligers van 50-70 jaar (n=312 CC, 378 CT en 125 TT), met een homocysteïne boven 13 µmol/l zoals vastgesteld tijdens screening, gedurende één jaar dagelijks gesuppleerd met foliumzuur (800 µg) of een placebo.

Resultaten: Bij de 'baseline' meting bedroeg de mediane folaatname 194 µg/dag en deze verschilde niet tussen de genotypen. Personen met het TT-genotype hadden een 13% lager serumfolaat en een 8% hoger homocysteïne dan personen met het CC-genotype. Bij een inname boven de 215 µg/dag (bovenste kwartiel) hadden personen met het TT-genotype een homocysteïne dat gelijk was aan dat van personen met de CC- of CT-genotypes die een inname hadden onder de 138 µg/dag (laagste kwartiel). Dit weerspiegelt een verhoogde folaatbehoefte van het TT-genotype. Na 1 jaar foliumzuur supplementie daalde het homocysteïnegehalte met 35% in de totale studiepopulatie. Vergeleken met de placebogroep ondergingen personen met het TT-genotype een homocysteïneverlaging van 4,3 µmol/l (-40%), en deze verlaging was significant groter (p<0.0001) dan die van personen met het CC-genotype (-3,2 µmol/l, -32%) of het CT-genotype (-3,0 µmol/l, -31%). Na 1 jaar supplementie bedroeg het gemiddelde homocysteïne van personen met de TT-, CT- en CC-genotypes respectievelijk 9,2; 9,6 en 9,7 µmol/l.

Conclusies: Personen met het TT-genotype hebben minimaal een 75-100 µg/dag hogere folaatname nodig om eenzelfde homocysteïne te bereiken als de CC- of CT-genotypes. Na 1 jaar 800 µg/dag foliumzuur supplementie bereikten personen met het TT-genotype een gelijke laagnormale homocysteïne als personen met de CC- of CT-genotypes.

Trefwoorden: methylenetetrahydrofolaatreductase; homocysteïne; folaatname; serumfolaat; rodebloedcelfolaat; ouderen