5,10-methylenetetrahydrofolate reductase common mutations, folate status and plasma homocysteine in healthy French adults of the Supplementation en Vitamines et Mineraux Antioxydants (SU.VI.MAX) cohort

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The 677cytosine (c) → thymine(T) mutation identified in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene has been frequently associated with an elevated plasma homocysteine concentration. The aim of the present study was to determine the impact of this MTHFR common mutation on plasma and erythrocyte folate (RCF) and plasma total homocysteine (tHcy) concentrations in healthy French adults. A cohort of 291 subjects living in the Paris area and participating in the Supplementation en Vitamines et Mineraux Antioxydants (SU.VI.MAX) study were analysed to assess the impact of MTHFR polymorphism 677C → T on folate status and plasma tHcy concentration. The frequency of the mutant homozygote for 677C → T polymorphism (677TT genotype) in the present cohort was 16·8 %. There were significant differences in plasma tHcy between 677CC, 677CT and 677TT genotype groups. The RCF concentrations were significantly different between each genotype, the lowest levels being associated with the 677TT genotype. When segregated by gender, no differences in tHcy between homozygous 677TT, heterozygous 677CT and wild-type 677CC genotype groups in women were observed. The fasting tHcy in women was unrelated to the 677C → T mutation. However, tHcy was significantly increased in men with the homozygous 677TT genotype. We also analysed the possible implication of a second new MTHFR polymorphism (1298A → C) in subjects with mild hyperhomocysteinaemia (4^{th} quartile of homocysteinaemia; tHcy >11·1 μmol/l). The polymorphism 1298A → C did not have a notable effect on tHcy or on the RCF levels. Our observations confirm a relatively high frequency of the 677TT genotype in the French population. Women with this genotype did not show the same increase in tHcy observed in men. In the present study dietary folate intake was not measured. Thus, the interaction of dietary folate with the MTHFR genotype in the French population needs further study.

Folate: Homocysteine: Methylenetetrahydrofolate reductase: Polymorphism

For some years, a special interest has been shown in the association between elevated plasma homocysteine and different forms of cardiovascular diseases (Boushey *et al.* 1995; Selhub, 1997). Homocysteine, methionine and cysteine are S-containing amino acids. Fasting levels of plasma total homocysteine (tHcy) mainly reflect the extent of homocysteine remethylation to methionine, whereas tHcy levels after a methionine load are a reflection of the

Abbreviations: A, adenine; C, cytosine; MTHFR, 5,10-methylenetetrahydrofolate reductase; RCF, erythrocyte folate; tHcy, total homocysteine; SU.VI.MAX, Supplementation en Vitamines et Mineraux Antioxydants; T, thymine.

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efficiency of the trans-sulfuration pathway (Miller *et al.* 1994). Some studies have shown that concentrations of plasma tHcy can be influenced by increased intake of B vitamins, particularly folic acid (Ubbink *et al.* 1994; Brönstrup *et al.* 1998; den Heijer *et al.* 1998; Brouwer *et al.* 1999).

Four key enzymes are involved in homocysteine metabolism: methylenetetrahydrofolate reductase (MTHFR); methionine synthase, which requires vitamin B_{12} as a coenzyme in the remethylation pathway; cystathionine β-synthase, which requires vitamin B_6 (pyridoxal 5′-phosphate) as a coenzyme in the trans-sulfuration pathway; and betaine–homocysteine *S*-methyltransferase, which can remethylate homocysteine in the liver and kidney.

The major cause of genetic predisposition to fasting mild hyperhomocysteinaemia is thought to be the presence of a thermolabile form of MTHFR (Harmon *et al.* 1996). MTHFR catalyses the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the major circulating form of folate and the methyl donor for the vitamin B_{12}-dependent remethylation of homocysteine to methionine (Rosenblatt, 1995). Thermolability of MTHFR is caused by a common missense mutation in the MTHFR gene with a cytosine (C) to thymine (T) substitution at nucleotide 677 (677C → T), which converts an alanine codon to a valine codon and reduces enzyme activity (Frosst *et al.* 1995). The 677C → T mutation is common in various populations, with reported homozygote frequencies of 0–38 % (allele frequency 2–44 %; Pepe *et al.* 1998; Schneider *et al.* 1998). Subjects who are homozygous for 677C → T have high plasma tHcy concentrations in the presence of folate insufficiency due to a 20 % decrease in MTHFR activity (Jacques *et al.* 1996). More recently, a second common variant in MTHFR (1298 adenine (A) → C) was observed in approximately 10 % of Dutch individuals (van der Put *et al.* 1998) and Canadian individuals (Weisberg *et al.* 1998) with a 1298CC genotype. This polymorphism, which converts a glutamic acid codon to an alanine codon, was also associated with decreased MTHFR activity. The combined heterozygosity for the 1298A → C and 677C → T mutations has been found to be associated with higher tHcy, decreased plasma folate concentrations and decreased MTHFR activity (van der Put *et al.* 1998; Weisberg *et al.* 1998).

The main objective of the present study was to determine the folate status and plasma homocysteine distribution in a subsample of healthy adults living in the Paris area and participating in the Supplementation en Vitamines et Mineraux Antioxydants (SU.VI.MAX) cohort, then to investigate the relationship between the two MTHFR common mutations and folate status and homocysteinaemia.

Subjects and methods

The SU.VI.MAX study started in 1994 and is designed to run for 8 years. It consists of 12 735 French subjects, with women in the age range 35–60 years and men in the age range 45–60 years (Hercberg *et al.* 1998). The study protocol was approved by the Ethical Committee for Studies with Human Subjects of the Paris-Cochin hospital and volunteers gave their informed consent for the investigation. Subjects were enrolled providing that they had no clinical evidence of cardiovascular disease. None of the enrolled subjects took vitamin or mineral supplements. Twelve percent of women and 18 % of men were current tobacco smokers, and 12 % of women were using oral contraceptives at the time of enrolment. Of the 804 subjects recruited in Paris, samples and data from 291 subjects collected between February and May 1997 were analysed for the present study. Fasting blood was collected by venipuncture. After collection, whole blood was kept at 4°C in the dark until it was centrifuged. The time elapsing between blood collection and plasma separation was less than 1 h (Hercberg *et al.* 1998). Aliquots were stored at −80°C until analysis. For erythrocyte folate (RCF) analysis, aliquots of whole-blood samples were diluted with 57 mmol ascorbic acid/l (1:11, v/v) and incubated for 1 h in a waterbath at 37°C for deconjugation of polyglutamyl folates by action of plasma pteroylglutamate hydrolase activity.

Folate concentrations were estimated by microbiological assay using *Lactobacillus casei* ATCC 7469 (*Lactobacillus rhamnosus*; Institut Pasteur, Paris, France) with folic acid–casei medium (Difco Labs, Detroit, MI, USA; Christides & Potier de Courcy, 1987). The CV for each assay (intra- and interassay) was <7 %. RCF values were calculated using packed cell volume measured by micro-centrifugation (Hoffbrand *et al.* 1966). Vitamin B_{12} was determined by an automated chemiluminescence method (Chiron Diagnostics, East Walpole, MA, USA). Pyridoxal 5′-phosphate was measured by HPLC and fluorimetric detection (Ubbink *et al.* 1986).

Plasma tHcy concentration (total amount of protein- and non-protein-bound homocysteine) was determined by HPLC and fluorimetric detection using a kit (BioRad Laboratories, Ivry sur Seine, France). Precolumn derivatisation of homocysteine by 4-(aminosulfonyl)-7-fluorobenzo-2-oxa-1,3-diazole was performed before HPLC separation and fluorescence detection. The chromatographic system consisted of a Kontron 422 HPLC pump (Kontron Instruments, Montigny Le Bretonneux, France), a Kontron 360 automated sample injector equipped with a 20 μl loop, an ODS (3·2 mm × 1 cm, 5 μm particle size) guard column (BioRad), and an ODS (3·2 mm × 7 cm, 3 μm particle size) reversed-phase column (BioRad). Detection of the 4-(aminosulfonyl)-7-fluorobenzo-2-oxa-1,3-diazole-derivatised homocysteine was achieved using a Jasco 821-FP spectrofluorimeter (Japan Spectroscopic Co, Tokyo, Japan). Excitation and emission wavelengths were set at 385 and 515 nm respectively. Concentrations were calculated according to a serum calibrator (BioRad).

Genomic DNA was isolated from peripheral blood leucocytes using a Genomix kit (Talent-Euromedex, Souffelweyersheim, France) according to the manufacturer's instructions. The polymerase chain reaction for the 677C → T mutation was performed according to the method of Frosst *et al.* (1995) and generated a 198 bp fragment using the forward primer 5′-TGA AGG AGA AGG TGT CTG CG and the reverse primer 5′-AGG ACG GTG CGG TGA GAG TG. The 677C → T mutation

Table 1. Plasma total homocysteine (tHcy); erythrocyte folate (RCF), and plasma folate, vitamin B_{12} and pyridoxal 5′-phosphate (PLP) concentrations in healthy French adults*

(Mean values and standard deviations and medians)

Variable	Mean	SD	Median	Percentile			
				10th	25th	75th	90th
All subjects (*n* 291)							
Plasma tHcy (μmol/l)	9·9	3·3	9·4	6·6	7·7	11·1	13·8
RCF (nmol/l)	582·5	190·7	547·0	367·7	455·6	688·9	838·9
Plasma folate (nmol/l)	16·4	11·2	13·4	5·9	9·8	20·1	28·1
Plasma vitamin B_{12} (pmol/l)	360·3	126·6	332·0	237·5	279·0	411·0	501·0
Plasma PLP (nmol/l)	61·5	35·9	55·0	29·4	38·5	72·2	93·4
Women (*n* 161)							
Plasma tHcy (μmol/l)	8·7	2·3	8·4	6·2	7·0	10·0	11·2
RCF (nmol/l)	592·0	198·9	542·5	384·1	451·0	712·8	856·7
Plasma folate (nmol/l)	17·3	10·9	14·8	6·0	10·2	22·3	30·7
Plasma vitamin B_{12} (pmol/l)	377·2	125·9	358·0	250·0	281·7	428·5	518·4
Plasma PLP (nmol/l)	59·8	42·1	51·6	28·1	36·9	68·7	88·95
Men (*n* 130)							
Plasma tHcy (μmol/l)	11·3	4·0	10·3	7·6	8·7	12·8	15·5
RCF (nmol/l)	570·7	180·0	565·2	336·0	457·4	670·8	824·0
Plasma folate (nmol/l)	15·2	11·4	12·9	5·9	9·1	18·6	24·3
Plasma vitamin B_{12} (pmol/l)	345·5	129·5	324·0	231·5	276·0	392·5	464·5
Plasma PLP (nmol/l)	62·4	28·3	58·8	30·8	44·7	73·2	95·8

* For details of subjects and procedures, see p. 892.

created a *Hinf*I recognition sequence with a cleavage product of 175 bp. Subjects in the highest quartile for tHcy were analysed for a possible effect of the second common mutation of the MTHFR gene, 1298A → C. The polymerase chain reaction for the 1298A → C mutation was carried out according to van der Put *et al.* (1998) in a total volume of 50 μl containing 1 μM forward primer 5′-CTT TGG GGA GCT GAA GGA CTA CTA C, 1 μM reverse primer 5′-CAC TTT GTG ACC ATT CCG GTT TG, 200 μM of each of the deoxynucleoside 5′-triphosphates, 1·5 units *Taq* polymerase (Appligène-Oncor, Iukirsh, France), and 5 μl *Taq* buffer. The polymerase chain reaction program on GeneAmp (Perkin Elmer, Foster, CA, USA) consisted of an initial denaturation step of 2 min at 94°C, followed by thirty-five cycles of 94°C (60 s), 51°C (60 s) and 72°C (30 s), and a final extension step at 72°C for 7 min. As the 1298A → C abolishes a *Mbo*II restriction site, mutations were investigated by polymerase chain reaction of genomic DNA and digestion with the *Mbo*II (Appligène-Oncor). Normal, heterozygote and mutant homozygote were identified as 677CC, 677CT and 677TT respectively for the 677C → T mutation and as 1298AA, 1298AC and 1298CC respectively for the 1298A → C mutation.

Statistical analyses were performed using StatView-5 (Statistical Analysis Institute Inc., Cary, NC, USA) on a MacIntosh computer. Results are expressed as mean values and standard deviations. Qualitative data were analysed using a Chi-squared test. Comparisons of quantitative data were analysed using ANOVA. As distributions of folate, vitamin B_{12} and pyridoxal 5′-phosphate were skewed, a log-transformation was used for statistical analysis. Significance was set at $P < 0·05$.

Results

The fasting plasma tHcy for all subjects was 9·9 (SD 3·3), range 4·4–39·0, median 9·4) μmol/l, with 6·6, 7·7, 11·1 and 13·8 μmol/l corresponding to the 10th, 25th, 75th and 90th percentiles respectively (Table 1). The mean RCF and plasma folate were 582·5 (SD 190·7) and 16·4 (SD 11·2) nmol/l respectively. Mean plasma vitamin B_{12} was 360·3 (SD 126·6) pmol/l. Mean plasma pyridoxal 5′-phosphate was 61·5 (SD 35·9) nmol/l.

The 677C → T MTHFR genotype distribution for all subjects is presented in Table 2. The frequencies of the homozygous 677TT, heterozygous 677CT, and wild-type 677CC genotypes were 16·8, 42·9 and 40·2 % respectively. There was a significant difference between 677CC and 677TT genotype groups for plasma tHcy ($P < 0·05$) but not between 677CT and 677CC groups. No significant differences were observed between the three genotypes for plasma folate and plasma pyridoxal 5′phosphate. There was a significant difference between 677CC and 677TT subjects for vitamin B_{12} but not between 677CT and 677CC subjects or between 677CT and 677TT subjects.

When subjects were divided into groups based on their genotype and gender, no differences between the three genotype groups for tHcy in women were observed (Table 2). However, in men tHcy was significantly higher for homozygous 677TT than heterozygous 677CT or wild-type 677CC ($P < 0·05$). Plasma tHcy was significantly higher in men than in women for the three genotypes ($P < 0·008$). There was a marked effect of RCF, but not plasma folate, on the association between genotype and tHcy in both women and men. RCF concentrations among 677TT genotypes were only 70 % of those among 677CC genotypes in men, compared with 80 % in women.

For the entire group, the 1st, 2nd, 3rd and 4th quartiles for tHcy concentration were 7·7, 9·4, 11·1 and 14·1 μmol/l respectively. Approximately 22 % of the subjects had a tHcy concentration of 11·2 μmol/l (4th quartile) and 5 % had tHcy concentrations in the range 15–20 μmol/l. Only one subject was moderately hyperhomocysteinaemic, with a tHcy concentration of 39 μmol/l. This subject was

Table 2. The effect of gender and 5,10-methylenetetrahydrofolate reductase genotype on plasma total homocysteine (tHcy), erythrocyte folate (RCF), and plasma folate, vitamin B_{12} and pyridoxal 5′-phosphate (PLP) concentrations in healthy French adults†

(Mean values and standard deviations)

Genotype …	677CC		677CT		677TT	
	Mean	SD	Mean	SD	Mean	SD
All subjects (*n* 291)						
n	117		125		49	
Percentage of total	40·2		42·9		16·8	
Gender and (F:M)	68:49		71:54		22:27	
Plasma tHcy (μmol/l)	9·8[a]	2·7	9·5[a]	2·8	11·2[b]	5·3
RCF (nmol/L)	639·2[a]	203·4	571·1[b]	170·0	477·8[c]	162·9
Plasma folate (nmol/l)	17·5	11·8	16·1	10·6	14·0	10·2
Plasma vitamin B_{12} (pmol/l)	379·6[a]	146·3	350·6[a,b]	110·4	338·4[b]	109·3
Plasma PLP (nmol/l)	65·2	41·4	59·9	35·2	56·5	20·0
Women (*n* 161)						
n	68		71		22	
Plasma tHcy (μmol/L)	9·0	2·4	8·5	2·3	8·4	1·8
RCF (nmol/)‡	638·8[a]	227·0	572·3[a,b]	167·7	514·1[b]	161·4
Plasma folate (nmol/l)	18·4	10·2	16·8	11·6	16·1	12·7
Plasma vitamin B_{12} (pmol/l)	393·4	130·5	370·9	123·9	346·6	116·5
Plasma PLP (nmol/l)	66·1[a]	48·2	56·6[b]	41·4	51·6[b]	15·9
Men (*n* 130)						
n	49		54		27	
Plasma tHcy (μmol/l)	10·6[a*]	2·4	10·7[a*]	2·9	13·8[b*]	6·6
RCF (nmol/l)‡	640·1[a]	162·5	571·1[b]	173·4	447·2[c]	161·0
Plasma folate (nmol/l)	16·8	14·1	15·2	9·9	12·2	7·7
Plasma vitamin B_{12} (pmol/l)	374·3	170·4	326·5	87·6	330·3	104·4
Plasma PLP (nmol/l)	65·3	31·5	64·1	28·2	53·6	21·1

F, female; M, male; C, cytosine; T, thymine.
[a,b,c]Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).
Mean values were significantly different from those of females: *$P \leq 0·0001$.
† For details of procedures, see p. 892.
‡ Mean values were not significantly different from those of females (Trend test).

homozygous for the 677C → T mutation and had an RCF concentration of 274·7 nmol/l.

We analysed the possible implication of the second polymorphism 1298A → C in mild hyperhomocysteinaemia, with particular attention to subjects in the 4th quartile of tHcy concentration. None of the subjects was homozygous for both mutations and none had the 677TT/1298AC or 677CT/1298CC genotypes (Table 3). In subjects without the 1298A → C mutation (i.e. subjects with the 1298AA genotype), increased RCF levels were associated with decreased tHcy concentration. RCF was lower in 677CC and 677CT subjects with the 1298AC genotype than in 677CC or 677CT subjects with the 1298AA genotype. However, the differences were not significant and the number of subjects in each subgroup was too small to expect a robust conclusion.

Discussion

In previous studies conducted in France up to 18·5 % of healthy control populations have been reported to be homozygous for the 677C → T mutation (Chadefaux-Vakemans *et al.* 1996;

Table 3. The effect of combined mutations at nucleotides 677 and 1298 in the 5,10-methylenetetrahydrofolate reductase gene on plasma total homocysteine (tHcy); erythrocyte folate (RCF) and plasma folate concentrations in French adults with mild hyperhomocysteinaemia (homocysteine >11·1 μmol/l)

(Mean values and standard deviations)

Genotype		*n*	M:F	tHcy (μmol/l)		RCF (nmol/l)		Plasma folate (nmol/l)	
				Mean	SD	Mean	SD	Mean	SD
1298AA	677CC	6	1:5	12·6	1·4	637·4	183·9	15·6	6·1
	677CT	14	4:10	14·1*	2·0	538·7	207·9	9·1*	3·6
	677TT	12	1:11	17·9*	7·1	396·3*	159·6	10·2	6·6
1298AC	677CC	14	4:10	13·1	1·4	571·1	180·2	12·0	5·7
	677CT	10	1:9	14·1	1·6	488·5	177·9	13·9	9·3
	677TT	0							
1298CC	677CC	7	2:5	13·8	1·3	597·9	157·3	10·9	6·8
	677CT	0							
	677TT	0							

F, female; M, male; A, adenine; C, cytosine; T, thymine.
Mean values were significantly different from those of subjects with the 1298AA/677CC genotype: *$P < 0·05$.
† For details of procedures, see p. 892.

Faure-Delanef *et al.* 1997). The consequences of this prevalence in the French population on tHcy and folate status requires clarification, because of the possible health complication of mild hyperhomocysteinaemia related to increased frequency of the 677TT genotype. The present investigation was carried out to try to establish the relationship between MTHFR common mutations in healthy French adults participating in the SU.VI.MAX cohort, and folate status and mild hyperhomocysteinaemia.

Mean fasting tHcy concentrations of all subjects in the present study are in accordance with previous observations; homozygosity for the mutation, and to a lesser extent heterozygosity, were associated with moderately increased fasting tHcy levels (Jacques *et al.* 1996; Christensen *et al.* 1997; Clarke *et al.* 1998). Low RCF levels were associated with increased tHcy levels in all subjects with a 677TT genotype. These findings confirm that the presence of thermolabile MTHFR is associated with lower RCF levels. Plasma folate concentration did not appear to be as good an indicator of folate status. This observation is in agreement with that of Molloy *et al.* (1997). Plasma folate levels are thought to reflect the day-to-day variations in dietary folate levels while RCF is a better indicator of long-term tissue storage levels. Folate was analysed by microbiological assay in the present study. Using microbiological assay, Molloy *et al.* (1998), Zittoun *et al.* (1998) and Brouwer *et al.* (1999) also found a low RCF associated with the 677TT genotype. These observations show the impact of this mutation on tissue folate levels, in contrast to the 1298A → C mutation which did not have a notable effect (Table 3).

Some studies have shown that homocysteine levels in the circulation, found in a general population, vary with gender (Brattström *et al.* 1994). Our results are in agreement with that observation. Based on the gender distribution, tHcy concentrations in women in the present study were similar for the three 677C → T MTHFR-genotype groups. It is interesting to note that the RCF level in 677TT females in the present study is comparable with the RCF level in 144 healthy Dutch females aged 18–40 years who were treated for 4 weeks with 500 μg folic acid/d (Brouwer *et al.* 1999). During the 4-week intervention RCF levels increased to 508 (SD 176) from 400 (SD 107) nmol/l at week 0. The lack of association between tHcy levels and 677C → T MTHFR genotypes may be because RCF concentrations were not at a deficient level. As folate supplementation may be useful in preventing hyperhomocysteinaemia in homozygous mutant individuals, the results of the present study suggest that nutritional habits may contribute to keeping tHcy levels low in French females.

The 1298A → C mutation has been shown to reduce MTHFR activity (Weisberg *et al.* 1998). In previous studies, we (Chango *et al.* 2000) and other researchers (van der Put *et al.* 1998; Weisberg *et al.* 1998) have observed that tHcy levels are increased, but not significantly, by the 1298A → C mutation in healthy subjects. In the present study, the polymorphism 1298A → C did not have a notable effect on the tHcy levels of subjects with mild hyperhomocysteinaemia (tHcy in the 4th quartile). Mild hyperhomocysteinaemia was more related to the MTHFR genotype.

Lifestyle factors such as smoking and high coffee consumption have been shown to be important determinants of the tHcy concentration in plasma (Nygard *et al.* 1997). High folate intakes or status have also been reported to prevent mild hyperhomocysteinaemia due to lifestyle factors (Nygard *et al.* 1998). The suggestion that the French eat more foods with a high folate content, such as fruits and vegetables which they buy and consume fresh, may be an explanation (Parodi, 1997). Men with the homozygous 677C → T mutation seem to be more sensitive to a decrease in RCF levels. The relatively mild hyperhomocysteinaemia may be related to their increased muscle mass, which requires more folate for homocysteine remethylation to methionine. This hypothesis is supported by the requirement for muscle creatine synthesis in the liver, which is by far the greatest consumer of *S*-adenosylmethionine in the body.

In conclusion, our observations confirm a relatively high frequency of the 677TT genotype in the French population and an association with elevated tHcy concentration in men but not in women. The reason why women with the 677TT genotype do not show the same increase in plasma tHcy concentration observed in men requires further clarification. Dietary folate intakes by our subjects were not measured; therefore, further studies with a larger sample size that take into account folate intake, geographical distribution and lifestyle factors are currently underway.

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