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The 677C → T mutation in the methylenetetrahydrofolate reductase gene: associations with plasma total homocysteine levels and risk of coronary atherosclerotic disease

Petra Verhoef ^{a,*}, Frans J. Kok ^a, Leo A.J. Kluijtmans ^b, Henk J. Blom ^b, Helga Refsum ^c, Per M. Ueland ^c, Dick A.C.M. Kruyssen ^d

^a Department of Epidemiology and Public Health, Agricultural University, P.O. Box 238, 6700 AE Wageningen, The Netherlands

^b Department of Pediatrics, University Hospital Nijmegen, Nijmegen, The Netherlands

^c Department of Clinical Biology, Division of Pharmacology, University of Bergen, Bergen, Norway

^d Zuiderziekenhuis Hospital, Rotterdam, The Netherlands

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Abstract

Homozygosity for a 677C → T mutation at the locus that codes for 5,10-methylenetetrahydrofolate reductase (MTHFR), a folate-dependent crucial enzyme in homocysteine metabolism, may render the enzyme thermolabile and less active and has been associated with increased levels of plasma total homocysteine (tHcy). We assessed whether this mutation was associated with increased risk of coronary atherosclerosis and plasma levels of tHcy and furthermore studied whether folate status would modify the associations. Data were collected from subjects with substantial coronary atherosclerosis ($\geq 90\%$ occlusion in one and $\geq 40\%$ occlusion in a second coronary artery, referred to as cases, $n = 131$) or virtually no coronary narrowing (referred to as coronary controls, $n = 87$) and from a population-based control group ($n = 100$), all residing in the Rotterdam area, The Netherlands. Both males and females, aged 25–65 years were studied. The frequency of homozygosity for the mutation (+ / +) in cases (10.0%) did not significantly differ statistically from that observed in coronary controls (11.5%, $P = 0.71$), population-based controls (7.0%, $P = 0.43$), or combined control groups (9.1%, $P = 0.80$). In the overall group (as well as in the three subgroups), plasma tHcy levels, fasting and to a lesser extent after a methionine-loading test, were higher in + / + subjects than in homozygous normal subjects (– / –), whereas heterozygous subjects (+ / –) had intermediate levels ($P_{\text{trend}} = 0.001$). The + / + subjects with erythrocyte folate levels < 790 nmol/l (population median) had a 77% (95% CI, 27–144%) higher geometric mean fasting tHcy (21.4, $\mu\text{mol/l}$) than those with higher erythrocyte folate (12.1 $\mu\text{mol/l}$). The odds ratio (OR) of coronary atherosclerosis for + / + subjects, with + / – and – / – subjects as the reference group, in analyses with combined control groups, was 1.1 (95% CI, 0.5–2.4). The ORs were 2.2 (95% CI, 0.7–6.8) and 0.6 (95% CI, 0.2–1.7) among subjects with low and high folate levels, respectively. Our study indicates that homozygosity for the 677C → T MTHFR mutation, especially in combination with low folate status, predisposes to high plasma levels of fasting tHcy. However, homozygosity for this mutation, whether or not in combination with low folate status, was not associated with increased risk of coronary artery disease. © 1997 Elsevier Science Ireland Ltd.

Keywords: Methylenetetrahydrofolate reductase; Coronary atherosclerosis; Missense mutation; Homocysteine; Folate status

1. Introduction

Elevated plasma total homocysteine (tHcy), an independent risk factor for atherosclerotic vascular disease [1,2], is partly genetically determined [3,4]. Homocys-

* Corresponding author.

teine is formed from methionine and is either catabolized in the vitamin B₆-dependent transsulfuration pathway or remethylated to methionine. This latter reaction is catalyzed by the enzyme methionine synthase, which requires 5-methyltetrahydrofolate (5-methyl-THF) as substrate and vitamin B₁₂ as cofactor. 5-Methyl-THF is formed by the reduction of 5,10-methylene-THF by 5,10-methylene-THF reductase (MTHFR), which is a regulating enzyme in homocysteine metabolism [5]. Consequently, deficiencies of MTHFR may result in elevation of plasma tHcy.

In 1988, Kang et al. [6,7] discovered a variant of the MTHFR enzyme, characterized by a specific enzyme activity of approximately 50% of the normal activity. The enzyme appeared to be thermolabile, providing an opportunity to distinguish between this variant and the normal enzyme. Furthermore, they and others described that thermolability of MTHFR was common in the normal population (5%), associated with raised tHcy levels, and increased risk of coronary artery disease [8–10]. Recently, a 677C → T mutation was detected in the MTHFR gene, and homozygosity for this mutation was associated with decreased specific enzyme activity, increased thermolability, and elevated tHcy [11], mainly in subjects with low levels of plasma folate [12,13]. Several investigations have now studied whether homozygosity for the MTHFR 677C → T mutation is a risk factor for cardiovascular disease. Three studies found a two- to threefold increased risk for atherosclerotic vascular disease [14–16], one study found a twofold increased risk for thrombotic vascular disease [17], but seven other studies observed virtually no association or even a slight inverse association between homozygosity for the mutation and risk of cardiovascular disease [18,24].

In the present study, we investigated the frequency of 677C → T genotype and its association with tHcy levels before and after methionine loading in subjects with severely occluded coronaries and normal coronaries, and in controls from the general population. Since an adequate folate status may counterbalance the defective production of 5-methyl-THF in subjects with thermolabile MTHFR, we also studied the interactive effect of genotype and erythrocyte folate status on plasma tHcy level and on risk of coronary atherosclerosis.

2. Patients and methods

2.1. Study population

A case-control study was conducted from June 1992 to June 1994. Cases and one control group were selected from patients aged 25–65 years, who underwent coronary angiography in the Zuiderziekenhuis Hospital in Rotterdam, the Netherlands. Subjects with either

severe coronary occlusions (referred to as cases) or without substantial coronary occlusions (referred to as coronary controls) were included. A second control group was drawn from the general population and comprised subjects with no history of cardiovascular disease (referred to as population-based controls). Exclusion criteria for all groups were diabetic, renal, hepatic, thyroid or gastro-intestinal disease, cancer, alcohol or drug abuse and psychiatric illness.

At angiography, projections were made of the major coronary vessels using standard catheterization techniques. A team of cardiologists reviewed the projections and prepared angiography reports. Based on these, a trained research nurse selected potential cases and coronary controls. Cases were defined as those having $\geq 90\%$ occlusion in one and $\geq 40\%$ occlusion in one additional coronary artery. Notably, 77.1% of the cases had $\geq 70\%$ occlusion in a second vessel. Coronary controls were defined as those having $\leq 50\%$ occlusion in only one coronary artery. The majority (79.5%), had no substantial coronary narrowing in all three arteries, whereas only 5.7% of them had 50% stenosis in a single coronary vessel. Thus, there was a marked contrast between cases and coronary controls, reducing the possibility of disease misclassification.

The conditions that led to angiography were mainly angina pectoris, whereas in some coronary controls a known valve defect was the reason. A total of 67 (51.1%) cases and six (6.9%) coronary controls had a history of myocardial infarction prior to angiography. In the coronary controls, the myocardial infarctions were due to coronary spasms or other nonatherosclerotic causes.

During the study period, a total of 2659 patients underwent coronary angiography. Of these, 2292 were not included mainly because of age, over 65 years ($n = 1122$), coronary occlusion outside ranges of case and control definition ($n = 486$), or the presence of one or more other exclusion criteria. Of the 367 subjects that were invited, 353 (96.2%) could be reached and of those 222 were willing to participate (131 cases, 91 coronary controls, response rate of 62.9%). Three of the 91 coronary controls that had originally participated, were excluded from analysis, as their angiography reports mentioned too much coronary narrowing at second evaluation (but not enough to be included in the study as a case). Furthermore, genotyping was not performed for one coronary control subject, leaving 87 coronary controls for analyses.

We obtained a population-based control group, from a register of about 10 000 men previously, randomly, sampled from the catchment area of cases for participation in a cholesterol-lowering trial (which was not conducted). Among men with no prior history of cardiovascular disease or diabetes, a random sample of 152 were invited for participation. Fifteen could not be

reached, 14 did not meet the inclusion criteria and 47 were not interested, leaving 76 study subjects for participation (response rate of 61.8%). One participant was excluded from analysis because he reported diabetes at the interview. Spouses of 45, randomly chosen, male participants were invited to participate, of whom 12 were not eligible, seven were not willing to participate, leaving 26 women for participation (response rate of 78.8%). Thus, a total of 101 population-based control subjects was studied. However, genotyping was not performed for one subject, leaving 100 population-based controls for analyses. All participants gave their written informed consent. The study protocol was approved by the medical ethics committee.

2.2. Blood sampling and examination

At the day of the examinations, venous blood samples were obtained from all subjects between 08:30 and 09:30 h, after a 10–12 h fast. L-methionine (0.1 g/kg body weight) mixed with orange juice was given orally, together with a standardized low protein breakfast. After breakfast, subjects were interviewed about medication, smoking habits, alcohol consumption and other coronary risk factors. Subjects received a standardized low protein lunch and were asked not to consume any protein containing foods, like milk, cheese or meat. At 6 h after methionine administration, a second blood sample was drawn for estimation of plasma tHcy in response to methionine provocation. From one population-based control subject, we did not obtain a blood sample after methionine loading.

Duplicate blood pressure readings were taken before and after the methionine loading test with the subject seated after 5 min rest. Height and weight (without shoes and heavy clothing) were measured in the morning.

For measurement of whole blood folate, 200 μ l of EDTA blood was mixed with 4 ml (1:20) freshly prepared 1% (w/v) ascorbic acid solution. The rest of the EDTA blood, to be used for measurement of tHcy and creatinine in plasma, was placed on ice immediately and in the dark, and centrifuged at 4°C within 1 h. Serum was obtained for measurement of total and HDL cholesterol and triglycerides.

2.3. Biochemical analyses and genotyping

Plasma tHcy, which refers to the sum of protein-bound, free oxidized and reduced species of homocysteine in plasma, was determined by a modification of the method of Refsum et al. [25]. Folate in whole blood was determined by radio immunoassay (Diagnostic Products Corporation, Dual-count solid phase, no boil assay) by Mimelab-Ab, Soraker, Sweden. We expressed folate concentration per haematocrit, referred to as

erythrocyte folate. Folate values were missing in two cases and two coronary controls. Creatinine, total cholesterol and HDL cholesterol (after precipitation of LDL and VLDL) were determined with enzymatic photometry.

DNA was obtained from the buffy coat of EDTA blood [26]. The mutation involves a C to T mutation at nucleotide 677, which converts an alanine to a valine residue. The alteration creates a *Hin*FI site, which was used for mutation analysis. The method has been described in detail elsewhere [11]. We used the symbols +/+, +/-, and -/- to refer to subjects who were homozygous for the mutation, heterozygous for the mutation, and homozygous for the wildtype, respectively.

2.4. Definition of variables and statistical analyses

Current smoking was defined as the use of any tobacco at the time of catheterization (cases) or on the day of methionine loading (controls). Subjects were defined as hypertensive with a systolic blood pressure \geq 160 mmHg or diastolic blood pressure \geq 95 mmHg, or when they were using anti-hypertensive medication. Hypercholesterolemia was defined as serum cholesterol \geq 6.5 mmol/l or the use of cholesterol-lowering drugs.

Differences in age, gender, and various other cardiovascular risk factor levels (adjusted for age and gender) between cases and each of the two control groups were tested for significance with Student's *t*-test for continuous variables and Pearson's χ^2 test for frequency measures. To study whether the frequency of +/+ genotype differed among the cases and the control groups, we used Pearson's χ^2 test. Furthermore, using logistic regression analysis, we calculated odds ratios (ORs) and 95% confidence intervals (CIs) for +/+ subjects, with +/- and -/- subjects as the reference group. We performed these analyses both with the separate control groups and with all controls combined. Plasma tHcy showed positive skewness, therefore log-transformation was applied. We compared geometric mean tHcy levels among the different genotypes, both in the overall study population and in subgroups of cases and both control groups. Differences between the -/ genotype and both +/+ and +/- genotypes were tested with Student's *t*-test. Tests for trend were performed with linear regression analysis. Calculation of ORs and of tHcy differences between the genotypes were repeated by strata of erythrocyte folate levels. We chose the median erythrocyte level (790 nmol/l) of the entire study population as a cutoff-point for the folate strata. This cutoff-point would leave us with comparable statistical power in both subgroups. The sample was too small to allow for a more meaningful cutoff-point, e.g. the 20th percentile. All reported *P*-values are for two-tailed tests.

Table 1
Characteristics and plasma total homocysteine levels

	Cases (<i>n</i> = 131)	Coronary controls (<i>n</i> = 87)	Population controls (<i>n</i> = 100)
Age (years)	52.5 ± 7.5	48.1 ± 8.0*	49.9 ± 6.9**
Gender (% male)	84.7	59.8*	74.0
Body mass index (kg/m ²)	26.6 ± 3.0	26.3 ± 3.5	26.0 ± 3.6
Total cholesterol (mmol/l)	6.8 ± 1.4	6.8 ± 1.7	6.7 ± 1.7
Total/HDL cholesterol	6.8 ± 2.0	6.2 ± 2.4*	6.0 ± 2.7**
Hypercholesterolemic (%)	67.2	62.5	49.5**
Triglycerides (mmol/l)	2.0 ± 1.3	1.7 ± 1.0*	1.4 ± 0.9**
Systolic blood pressure (mmHg)	134 ± 13	133 ± 16	135 ± 14
Diastolic blood pressure (mmHg)	81 ± 8	79 ± 9*	82 ± 8
Hypertensive (%)	83.2	53.4*	16.8**
Pack years	29.8 ± 27.1	25.2 ± 20.3*	21.9 ± 21.4**
Alcohol consumption (glasses/day)	0.8 ± 1.3	1.1 ± 1.7	1.3 ± 1.6**
Fasting tHcy (μmol/l)	12.7	11.6	11.9
Post-load tHcy (μmol/l)	39.4	36.5*	37.3
Increase in tHcy (μmol/l)	26.1	24.4	25.1

tHcy, total homocysteine.

* $P < 0.05$, cases versus coronary control subjects, adjusted for age and gender (with exception of these variables themselves).

** $P < 0.05$, cases versus population-based control subjects, adjusted for age and gender (with exception of these variables themselves).

Tested with Student's *t*-test for continuous variables (geometric means are shown for tHcy, mean ± S.D. are shown for other variables) and Pearson's χ^2 test for frequencies. For tHcy, testing was performed with log-transformed variables.

3. Results

3.1. Characteristics and tHcy

Age, gender, coronary risk factors and plasma tHcy levels of the cases and the two control groups are shown in Table 1. Except for age and gender, all other risk factors were adjusted for age and gender. In comparison with each of the control groups, cases had a statistically significant higher mean age, proportion of hypertensive subjects, mean level of total/HDL cholesterol, mean serum level of triglycerides and mean pack years of smoking. In addition, cases had a statistically significant higher proportion of males and mean diastolic blood pressure, compared to coronary controls. Furthermore, compared to population-based controls, cases has a statistically significant higher proportion of hypercholesterolemic subjects and a lower mean alcohol consumption. The mean plasma level of creatinine was slightly (but not statistically significant) higher in cases (data not shown).

Geometric means of age- and sex-adjusted plasma levels of tHcy were higher in cases than in any of the two control groups (Table 1). However, the difference was statistically significant only for post-load tHcy in the comparison with coronary controls. To increase statistical power, we combined both control groups. Cases had 9% higher geometric mean fasting tHcy levels ($P = 0.02$) and 7% higher geometric mean post-load tHcy levels ($P = 0.04$) than the combined control groups, after adjusting for age and gender. The geometric mean of increase after loading (i.e. post-load minus fasting level), was 6% ($P = 0.15$) higher in cases relative to the combined control groups.

3.2. MTHFR genotype

In the total study population, the allele frequency of the mutation was 33%. The frequency of homozygosity for the mutation (+/+) in cases (10.0%) did not statistically differ significantly from that observed in coronary controls (11.5%, $P = 0.71$), population-based controls (7.0%, $P = 0.43$) or combined control groups (9.1%, $P = 0.80$) (Table 2).

Gender ratio, age, serum creatinine and risk factors for coronary artery disease (body mass index, alcohol consumption, serum levels of total/HDL cholesterol, triglycerides, hypertension and pack years of smoking) were not materially nor significantly different statistically between the genotype subgroups, in any of the three study groups, nor in the total study population.

3.3. Levels of tHcy by MTHFR genotype

Geometric mean levels of tHcy were highest among the +/+ subjects and lowest among the -/- subjects. The +/- individuals had slightly elevated levels, compared to -/- subjects (Table 3). This was true for all three measurements of tHcy, both in cases and each of the control groups, with the exception of tHcy increase after methionine loading in population-based control subjects, where the geometric mean tHcy level was slightly higher in +/- subjects than in +/+ subjects, although both groups had higher geometric mean levels than -/- subjects. Ratios of geometric mean tHcy levels of +/+ subjects to levels of -/- subjects were highest for fasting tHcy and lowest for the increase in tHcy after methionine loading; when

Table 2
Frequencies of MTHFR genotype

Genotype	Cases (<i>n</i> = 131)	Coronary controls (<i>n</i> = 87)	Population controls (<i>n</i> = 100)
Homozygous normal (–/–)	45.0 (59)	39.1 (34)	45.0 (45)
Heterozygous mutant (+/–)	45.0 (59)	49.4 (43)	48.0 (48)
Homozygous mutant (+/+)	10.0 (13)	11.5 (10)	7.0 (7)

combining all subjects, these ratios were 1.36 (i.e. $15.5 \div 11.4$) for fasting tHcy, 1.25 (i.e. $44.2 \div 35.4$) for post-load tHcy, and 1.18 (i.e. $27.7 \div 23.6$) for post-load increase in tHcy. In the combined study groups, tests for linear trend were statistically significant, for all three tHcy measurements.

3.4. MTHFR genotype and risk of coronary atherosclerosis

Since elevation of tHcy was much more obvious for +/+ subjects than +/– subjects, in accordance with other publications [11,16,21], we considered –/– and +/– subjects as one reference group (i.e. OR = 1.0). The ORs for +/+ genotype were 0.9 (95% CI, 0.4–2.0) and 1.5 (95% CI, 0.6–3.8), with coronary controls and population-based controls as referents, respectively. When performing analyses with combined control groups, the OR for +/+ genotype was 1.1 (95% CI, 0.5–2.3). The corresponding multivariate adjusted OR (i.e. adjusted for age, gender, body mass index, alcohol consumption, serum levels of total/HDL cholesterol and triglycerides, hypertension, serum creatinine and

pack years of smoking) for +/+ genotype was 1.2 (95% CI, 0.5–3.2).

3.5. Effect modification by erythrocyte folate

Erythrocyte folate did not materially nor statistically significantly differ between the genotypes, neither in the separate study groups, nor in the pooled study groups (data not shown). Using combined data of the entire study population, we calculated plasma tHcy levels of groups with low and high erythrocyte folate, per genotype, as shown in Fig. 1. Geometric mean plasma tHcy was higher for the subjects with low compared to those with high erythrocyte folate, especially in the +/+ group. As Fig. 1 is merely a demonstration, Table 4 shows mean levels of tHcy for individuals with low and high folate status in the +/+ group. The relation was most evident for fasting tHcy, as shown in Fig. 1 and Table 4.

Table 3
Geometric mean plasma total homocysteine by MTHFR genotypes

	Cases (<i>n</i> = 131)	Coronary controls (<i>n</i> = 87)	Population controls (<i>n</i> = 100)	All subjects (<i>n</i> = 318)
<i>μ</i> mol/l				
Fasting tHcy				
–/–	12.4	10.3	10.9	11.4
+/–	12.7	11.9**	12.1**	12.3**
+/+	16.6	13.1	17.4	15.5*** <i>P</i> = 0.001 ^a
Post-load tHcy				
–/–	36.4	34.5	34.7	35.4
+/–	41.3**	36.8	38.9**	39.2**
+/+	46.4	42.0***	43.6	44.2*** <i>P</i> = 0.001
Increase in tHcy				
–/–	23.6	23.7	23.6	23.6
+/–	27.9**	24.4	26.5	26.4**
+/+	28.4	28.1	25.8	27.7*** <i>P</i> = 0.002

tHcy, total homocysteine.

^a Test for linear trend.

** *P* < 0.05 for (+/–) versus (–/–); *** *P* < 0.05 for (+/+) versus (–/–).

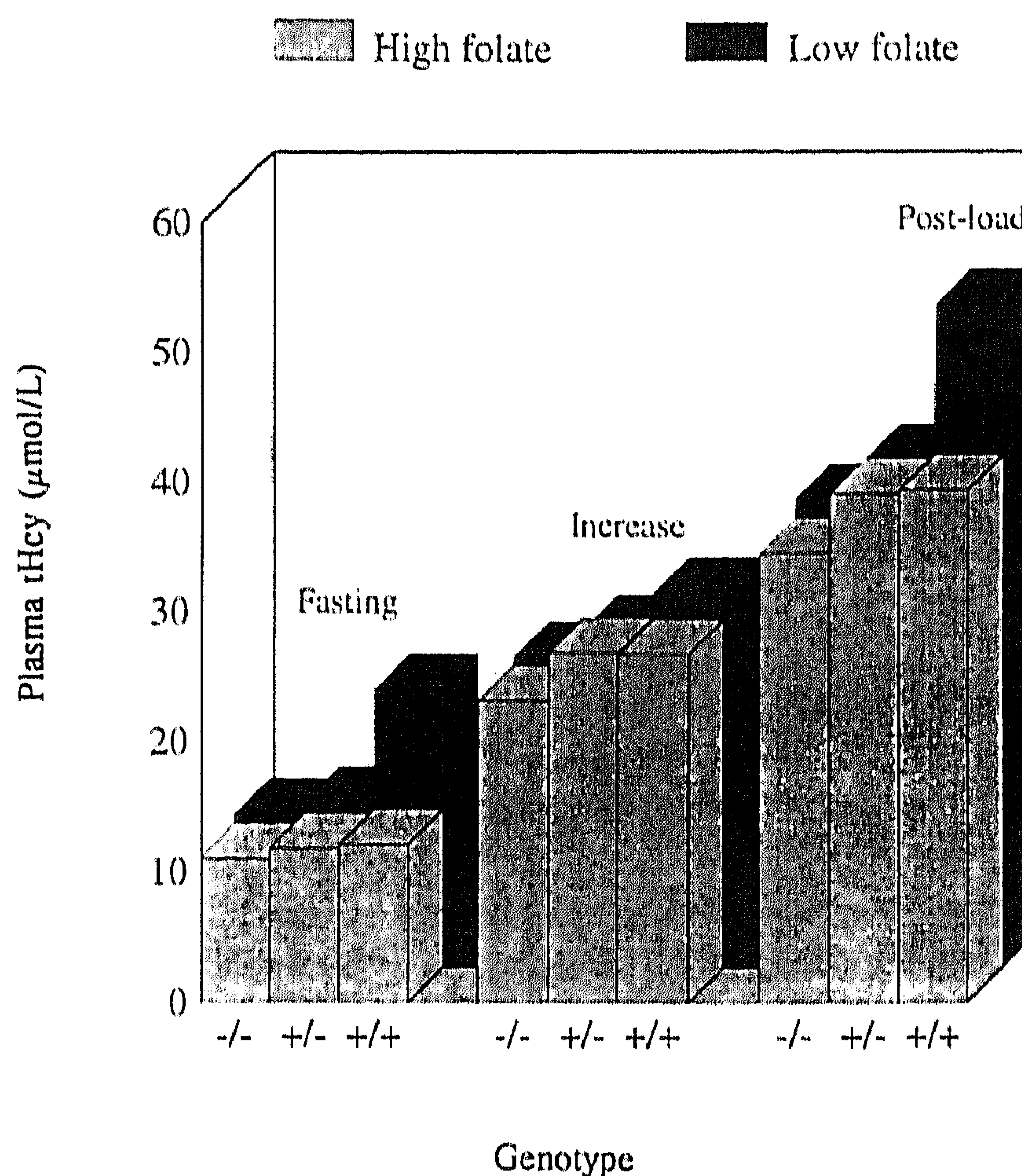


Fig. 1. Plasma total homocysteine (tHcy) for genotypes of 677C → T MTHFR mutation, stratified by low and high erythrocyte folate (cut off-point 790 nmol/l), in total study population.

Table 4

Plasma total homocysteine among subjects homozygous for 677C → T MTHFR mutation, stratified by median erythrocyte folate level (790 nmol/l)

	Low folate (<i>n</i> = 13)	High folate (<i>n</i> = 17) μmol/l	Difference in geometric means Percent (95% CI)
Fasting tHcy			
Geometric mean	21.4	12.1	77 (27–144%)
Mean ± S.D.	25.7 ± 18.2	12.6 ± 3.3	
Post-load tHcy			
Geometric mean	51.2	39.5	30 (–2–70%)
Mean ± S.D.	56.3 ± 26.2	41.6 ± 15.4	
Increase in tHcy			
Geometric mean	28.9	26.8	8 (–18–42%)
Mean ± S.D.	30.6 ± 10.5	29.1 ± 14.0	

CI, confidence interval.

Finally, using combined control groups, we calculated ORs of coronary atherosclerosis for +/+ subjects relative to +/- and -/- subjects, for strata of low and high erythrocyte folate level. The ORs were 2.2 (95% CI, 0.7–6.8) and 0.6 (95% CI, 0.2–1.7) among subjects with low and high folate levels, respectively. However, the interaction was not statistically significant; the *P*-value for the interaction term of folate status and +/+ genotype was 0.11 and the logistic regression model including genotype, folate status and the interaction term did not significantly increase the log likelihood ratio estimate in comparison to a model including only genotype and folate status. The ORs were not materially affected by multivariate adjustment for age, gender and other major coronary risk factors, but CIs widened substantially.

4. Discussion

In the present investigation, we showed that homozygosity for the 677C → T mutation in the MTHFR gene was associated with raised plasma tHcy levels, particularly when measured in the fasting state. However, +/+ genotype was not associated with increased risk of severe coronary atherosclerosis. Elevation of tHcy in +/+ subjects was limited to those with erythrocyte folate levels below the median of the study population. However, we have not been able to prove the hypothesis that the combination of +/+ genotype and low folate status infers an increased risk of coronary artery disease.

Our observation of elevation of tHcy levels in +/+ subjects compared to -/- and +/- subjects is supported by other studies [11–14,16,21,27]. In our study, as well as in others that studied the relation of the mutation with both fasting and post-load tHcy levels [11,14,16], the trend was stronger for fasting than for post-load levels. These findings support the idea that fasting tHcy level is to a large extent determined by

homocysteine remethylation, whereas increased post-load tHcy primarily reflects abnormalities in the transsulfuration pathway [28,29]. The only other study [12] that also studied the relationship of the mutation with increase in tHcy after methionine loading (i.e. after subtracting fasting level, which in fact is a better reflection of transsulfuration than absolute post-load tHcy level), observed no differences between the genotypes. In contrast with their finding, we observed a statistically significant 18% higher geometric mean of increase in tHcy after methionine loading in +/+ subjects compared to -/- subjects (Table 3). Whether this is a chance finding or whether this indicates an effect of the mutation on homocysteine transsulfuration needs to be determined in future studies.

Other studies have observed that the trend of higher tHcy levels among +/+ subjects was only apparent in those with low folate levels in plasma or serum [12,13,21]. However, those findings might still have been due to an artefact, as +/+ genotype is associated with reduced plasma folate concentration (the main circulating form of folate in plasma is 5-methyl-THF) [13,20,21,27]. As there is still some variation in MTHFR activity within the group of +/+ subjects [11,16], it is not unlikely that +/+ subjects at the low end of the range of enzyme activity will have both low concentrations of plasma folate and high concentrations of tHcy (and those at the high end of enzyme activity will vice versa have both high folate and low tHcy). We observed no difference in erythrocyte folate levels among the various genotypes, but we found a dose-response relation between 677C → T genotype and plasma tHcy. Furthermore, we observed higher fasting tHcy levels among subjects with low compared to high erythrocyte folate, which is in agreement with results published by others [30]. Thus, our data suggest that cellular folate status and MTHFR activity have both independent as well as interactive relations with plasma tHcy. Erythrocyte folate is a measure of tissue folate stores, which reflects the balance between folate intake,

absorption, metabolism, and utilization [31]. Provided that the determinants of folate homeostasis other than folate intake are similar within groups of genotype, our findings suggest that adequate intake is essential for maintaining low tHcy in +/+ subjects. Furthermore, our finding of higher post-load increase tHcy levels in +/+ subjects possibly shows that these individuals may also benefit from increasing intake of vitamin B₆, the coenzyme in the transsulfuration pathway.

Surprisingly, subjects with the +/+ genotype did not have increased risk of coronary atherosclerosis, despite elevated levels of tHcy, which is generally considered to be an independent risk factor for cardiovascular disease [1]. When including only coronary controls in the risk analyses, we observed an OR of 0.9 for +/+ genotype, compared to the -/- and +/- genotypes. With population-based controls, we estimated an OR of 1.5, but neither of the ORs was statistically significant nor did the ORs differ statistically significantly from each other. There was a fair contrast between cases and coronary controls with respect to coronary artery narrowing, but in the coronary controls we had no information on the occurrence of thrombi leading to angina without coronary artery narrowing. Assuming that these were common and related to MTHFR genotype, this may have attenuated the association to some extent. In population-based controls, on the other hand, one is never sure about the extent of coronary artery narrowing, since these controls may have substantial, although not clinically manifest coronary atherosclerosis. This again may have lead to some attenuation of the association. However, the type of design we used, i.e. selecting cases and controls by extent of coronary narrowing, and additionally studying an unselected populationbased control group, has been applied by others and both those studies and ours have shown positive, independent associations between plasma tHcy and risk of coronary atherosclerotic disease [32–34].

Other epidemiologic studies that estimated risk of vascular disease associated with +/+ genotype show conflicting results. Kluijtmans et al. [16], the first ones to report on the association, found an OR of vascular disease of 3.1 (95% CI, 1.0–9.2) for +/+ genotype in comparison with +/- and -/- genotypes. The discrepancy between our present study and their study, both conducted in Dutch populations, may be explained by the fact that they selected young patients, with absence of hyperlipoproteinemia, hypertension and diabetes mellitus, i.e. individuals that were otherwise at a low risk of cardiovascular disease. A Japanese study [15], found a statistically significantly, twofold increased risk of myocardial infarction for +/+ genotype. An Irish study [14], showed a statistically significantly threefold increased risk of premature coronary artery disease. From data of a study conducted in Italy

[17], we derived an OR of 2.4 (95% CI, 1.3–4.6) for early onset venous or arterial occlusive disease or thrombosis occurring at unusual sites. Adams et al. [18], in a study of myocardial infarction conducted in the UK, found an OR of 0.9 (95% CI, 0.5–1.5). An Australian study by Wilcken et al. [24] used a study design similar to ours, comparing patients with and without angiographically documented coronary artery disease and healthy subjects. They found an OR of 1.0 (95% CI, 0.6–1.7) for the comparison of subjects with severe disease with those with no or mild coronary artery disease. Results were similar when those with severe coronary disease were compared to healthy subjects. A study conducted in the Boston area [23] found an OR of myocardial infarction of 1.1 (95% CI, 0.6–1.9). Ma et al. [21], in the Physicians' Health Study, observed an OR of myocardial infarction of 0.8 (95% CI, 0.5–1.3). With data of a study by Narang et al. [22], conducted in the UK, an OR of coronary heart disease of 0.6 (95% CI, 0.2–2.1) could be calculated. Findings of a French study [19] in subjects with non-insulin dependent diabetes mellitus yielded an OR of coronary heart disease of 0.7 (95% CI, 0.4–1.2). Finally, with data of a study conducted in the US [20], an OR of 0.5 (95% CI, 0.3–0.9) for peripheral and cerebrovascular disease could be calculated (we excluded Canadian neonates from the control group, but this did not materially affect the OR).

Summarizing results from the twelve studies (including ours) reported so far, eight of them did not find increased risk of cardiovascular disease for +/+ genotype, despite the fact that these subject generally have increased (fasting) tHcy levels, about 2–4 $\mu\text{mol/l}$ higher than in -/- or +/- subjects [11–13,16,21]. Based on a large meta-analysis [1], for this difference in plasma tHcy an OR of about 1.5 for cardiovascular disease would be expected for the +/+ genotype compared to others. The majority of studies found an estimate much lower than this, although many CIs included the value of 1.5, and +/+ genotype cannot be totally ruled out as a factor that exerts increased risk of cardiovascular disease through tHcy elevation. However, based on the currently available data, it is certainly not an important risk factor for cardiovascular disease.

It is not unlikely, however, that the +/+ genotype will only emerge as a risk factor for cardiovascular disease in a source population with a low folate status. This may explain why positive associations were observed in populations where use of vitamin supplements and fortification of food products with folic acid is uncommon (i.e. Dutch [16] or Irish population [14]), as opposed to no association in most US populations [20,21,23], where use of vitamin supplements and consumption of folate fortified cereals is more common. Nevertheless, the studies that investigated the associa-

tion of the mutation with risk of cardiovascular disease among subgroups of low and high folate intake [23], or plasma folate [21] did not show a significantly positive relationship among those with low intake or plasma levels of folate either. With our data we were not able to prove the hypothesis that +/+ genotype is a risk factor for coronary atherosclerotic disease among those with low erythrocyte folate levels either.

The absence of a positive association between +/+ genotype and risk of coronary heart disease may thus indicate that elevated plasma tHcy is not a risk factor for cardiovascular disease, but merely a marker of low folate status, which for example through prothrombotic action confers the actual risk, as recently suggested by results from an animal study [35]. Another explanation could be that the mutation has a protective effect, that mitigates against the adverse effect of elevated plasma tHcy. This aspect may need further study.

In conclusion, we have demonstrated a positive interaction between the 677C → T +/+ genotype and low erythrocyte folate, resulting in elevated plasma tHcy, especially in the fasting state. However, subjects who are homozygous for this common mutation, whether or not with a low folate status, did not have increased risk of coronary atherosclerosis. This absence of an association between the mutation and risk of cardiovascular disease, also observed in many other studies, either indicates that elevation of plasma tHcy is not a risk factor for cardiovascular disease, or that the mutation has a beneficial effect as well, which mitigates against the potential harmful effects of tHcy elevation. The results presented here suggest that increased dietary folate intake is a means to lower plasma tHcy levels in subjects who are homozygous for the MTHFR mutation, but subsequent effects on risk of coronary heart disease are uncertain.

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References

- [1] Boushey CJ, Beresford SAA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. *J Am Med Assoc* 1995;274:1049–57.
- [2] Selhub J, Jacques PF, Bostom AG, D'Agostino RB, Wilson PWF, Belanger AJ, O'Leary DH, Wolf PA, Schaefer EJ, Rosenberg IH. Association between plasma homocysteine concentrations and extracranial carotid-artery stenosis. *N Engl J Med* 1995;332:286–91.
- [3] Genest JJ Jr., McNamara JR, Upson B, Salem DN, Ordovas JM, Schaefer EJ, Malinow MR. Prevalence of familial hyperhomocyst(e)inemia in men with premature coronary artery disease. *Arterioscler Thromb* 1991;11:1129–36.
- [4] Wu LL, Wu J, Hunt SC, James BC, Vincent GM, Williams RR, Hopkins PN. Plasma homocyst(e)ine as a risk factor for early familial coronary artery disease. *Clin Chem* 1994;40:552–61.
- [5] Ueland PM, Refsum H, Brattstrom L. Plasma homocysteine and cardiovascular disease. In: Francis RB Jr (ed). *Atherosclerotic Cardiovascular Disease, Hemostasis and Endothelial Function*, New York: Marcel Dekker, 1992;183–236.
- [6] Kang SS, Wong PWK, Zhou J, Sora J, Lessick M, Ruggie N, Greevich G. Thermolabile methylenetetrahydrofolate reductase in patients with coronary artery disease. *Metabolism* 1988;37:611–3.
- [7] Kang SS, Zhou J, Wong PWK, Kowalysyn J, Strokosch G. Intermediate homocysteinemia a thermolabile variant of methylenetetrahydrofolate reductase. *Am J Hum Genet* 1988;43:414.
- [8] Kang SS, Wong PWK, Susmano A, Sora J, Norusis M, Ruggie N. Thermolabile methylenetetrahydrofolate reductase, an inherited risk factor for coronary artery disease. *Am J Hum Genet* 1991;48:536–45.
- [9] Kang SS, Passen EL, Ruggie N, Wong PW, Sora H. Thermolabile defect of methylenetetrahydrofolate reductase in coronary artery disease. *Circulation* 1993;88:1463–9.
- [10] Engbersen AMT, Franken DG, Boers GHJ, Stevens EMB, Trijbels FJM, Blom HJ. Thermolabile 5,10-methylenetetrahydrofolate reductase as a cause of mild hyperhomocysteinemia. *Am J Hum Genet* 1995;56:142–50.
- [11] Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJH, Den Heijer M, Kluijtmans LAJ, Van den Heuvel LP, Rozen R. A candidate genetic risk factor for vascular disease: A common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 1995;10:111–3.
- [12] Jacques PF, Bostom AG, Williams RR, Ellison RC, Eckfeldt JH, Rosenberg IH, Selhub J, Rozen R. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase and plasma homocysteine concentrations. *Circulation* 1996;93:7–9.
- [13] Harmon DL, Woodside JV, Yarnell JWG, McMaster D, Young IS, McCrum EE, Gey KF, Whitehead AS, Evans AK. The common 'thermolabile' variant of methylene tetrahydrofolate reductase is a major determinant of mild hyperhomocysteinemia. *Q J Med* 1996;89:571–7.
- [14] Gallagher PM, Meleady R, Shields DC, Tan KS, McMaster D, Rozen R, Evans A, Graham IM, Whitehead AS. Homocysteine and risk of premature coronary heart disease. Evidence for a common gene mutation. *Circulation* 1996;94:2154–8.
- [15] Izumi M, Iwai N, Ohmichi N, Nakamura Y, Shimoike H, Kinoshita M. Molecular variant of 5,10-methylenetetrahydrofolate reductase is a risk factor of ischemic disease in the Japanese population. *Atherosclerosis* 1996;121:293–4.
- [16] Kluijtmans LAJ, Van den Heuvel LPWJ, Boers GHI, Frosst P, Stevens EMB, Van Oost BA, Den Heijer M, Trijbels FJM,

- Rozen R, Blom HJ. Molecular genetic analysis in mild hyperhomocysteinemia: A common mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for cardiovascular disease. *Am J Hum Genet* 1996;58:35–41.
- [17] De Franchis R, Mancini FP, D'Angelo A, Sebastio G, Fermo I, De Stefano V, Margaglione M, Mazzola G, Di Minno G, Andria G. Elevated total plasma homocysteine and mutation in 5,10-methylenetetrahydrofolate reductase gene in thrombotic vascular disease. *Am J Hum Genet* 1996;58:262–4.
- [18] Adams M, Smith PD, Martin D, Thompson JR, Lodwick D, Samani NJ. Genetic analysis of thermolabile methylenetetrahydrofolate reductase as a risk factor for myocardial infarction. *Q J Med* 1996;89:437–44.
- [19] Brulhart M-C, Dussoix P, Ruiz J, Passa P, Froguel Ph, James RW. The (Ala-Val) mutation of methylenetetrahydrofolate reductase as a genetic risk factor for vascular disease in noninsulin-dependent diabetic patients. *Am J Hum Genet* 1997;60:228–9.
- [20] Deloughery TG, Evans A, Sadehgi A, McWilliams J, Henner WD, Taylor LM, Press RD. Common mutation in methylenetetrahydrofolate reductase. Correlation with homocysteine metabolism and late-onset vascular disease. *Circulation* 1996;94:3074–8.
- [21] Ma J, Stampfer MJ, Hennekens CH, Fross P, Selhub J, Horsford J, Malinow MR, Willett WC, Rozen R. Methylenetetrahydrofolate reductase polymorphism, plasma folate, homocysteine and risk of myocardial infarction in US physicians. *Circulation* 1996;94:2410–6.
- [22] Narang R, Callaghan G, Haider AW, Davies GJ, Tuddenham EGD. Methylenetetrahydrofolate reductase and coronary artery disease. *Circulation* 1996;94:2322–3.
- [23] Schmitz C, Lindpaintner K, Verhoef P, Gaziano JM, Buring J. Genetic polymorphism of methylenetetrahydrofolate reductase and myocardial infarction: A case-control study. *Circulation* 1996;94:1812–4.
- [24] Wilcken DEL, Wang XL, Sim AS, McCredie M. Distribution in healthy and coronary populations of the methylenetetrahydrofolate reductase (MTHFR) C667T mutation. *Arterioscler Thromb Vasc Biol* 1996;16:787–882.
- [25] Fiskerstrand T, Refsum H, Kvalheim G, Ueland PM. Homocysteine and other thiols in plasma and urine; automated determination and sample stability. *Clin Chem* 1993;39:263–71.
- [26] Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988;16:1215.
- [27] Van der Put NMJ, Steegers-Theunissen RPM, Frosst P, Trijbels FJM, Eskes TKAB, Van den Heuvel LP, Mariman ECM, Den Heijer M, Rozen R, Blom HJ. Mutated methylenetetrahydrofolate reductase as a risk factor for spine bifida. *Lancet* 1995;346:1070–1.
- [28] Brattstrom L, Israelsson B, Norrving B, Bergqvist D, Thorne J, Hultberg B, Hamfelt A. Impaired homocysteine metabolism in early-onset cerebral and peripheral occlusive arterial disease. Effects of pyridoxine and folic acid treatment. *Atherosclerosis* 1990;81:51–60.
- [29] Miller JW, Nadeau MR, Smith D, Selhub J. Vitamin B₆ deficiency vs folate deficiency: Comparison of responses to methionine loading in rats. *Am J Clin Nutr* 1994;59:1033–9.
- [30] Chadeaux B, Coper BA, Gilfix BM, Lue-Shing H, Carson W, Gavsie A, Rosenblatt DS. Homocysteine: Relationship to cobalamin, serum folate, erythrocyte folate and lobation of neutrophils. *Clin Invest Med* 1994;17:540–50.
- [31] Davidson S., Passmore R., Eastwood MA. *Human Nutrition and Dietetics*, 8th edn. Hong Kong: Longman Group Limited, 1986.
- [32] Ubbink JB, Vermaak WHJ, Bennett JM, Becker PJ, Van Staden DA, Bissbort S. The prevalence of homocysteinemia and hypercholesterolemia in angiographically defined coronary heart disease. *Klin Wochenschr* 1991;69:527–34.
- [33] Pancharuniti N, Lewis CA, Sauberlich HE, Perkins LL, Go RCP, Alvarez JO, Macaluso M, Acton RT, Copeland RB, Cousins AL, Gore TB, Cornwell PE, Roseman JM. Plasma homocyst(e)ine, folate and vitamin B₁₂ concentrations and risk of early-onset coronary artery disease. *Am J Clin Nutr* 1994;59:940–8.
- [34] Verhoef P. Homocysteine, B-vitamins and cardiovascular disease: Epidemiologic evidence. PhD thesis, Wageningen, Agricultural University, 1996.
- [35] Durand P, Prost M, Blache D. Pro-thrombotic effects of a folic acid deficient diet in rat platelets and macrophages related to elevated homocysteine and decreased *n*-3 polyunsaturated fatty acids. *Atherosclerosis* 1996;121:231–43.