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ORIGINAL ARTICLE

Folate, homocysteine levels, methylenetetrahydrofolate reductase (*MTHFR*) 677C → T variant, and the risk of myocardial infarction in young women: effect of female hormones on homocysteine levels

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Summary. In young women data are limited about the association between myocardial infarction (MI) and hyperhomocysteinemia, low folate or methylenetetrahydrofolate reductase (*MTHFR*) genotypes. The effect of oral contraceptive (OC) use on plasma homocysteine levels is not clear. We assessed the association between hyperhomocysteinemia, low folate, *MTHFR* 677TT mutation and risk of MI, and we investigated the effect of OC use on homocysteine levels in controls. In 181 patients with a first MI and 601 controls 18–49 years of age from a population-based case–control study, non-fasting blood samples were available. The homozygote mutant allele (TT) was detected in 12% of the patients and in 10% of controls. The odds ratio (OR) for MI in TT patients compared with the wild-type (CC) controls was 1.3 [95% confidence interval (CI) 0.8, 2.3]. For all *MTHFR* genotypes combined, the OR for MI in the lowest quartile of folate (<5.4 nmol L^{-1}) compared with the highest quartile (>10.4 nmol L^{-1}) was 3.0 (95% CI 1.7, 5.1). A 2-fold increased risk of MI was found in women with the TT genotype who had folate levels below the median of 7.4 nmol L^{-1} compared with CC genotype and folate levels above the median (OR =2.0; 95% CI 1.0, 3.7). Mean homocysteine levels were 12.2 μmol L^{-1} in OC users and 12.3 μmol L^{-1} in non-users. Only at the 97.5 percentile (cut-off 21.0 μmol L^{-1}) was the adjusted OR for higher vs. lower homocysteine levels increased by 2.8-fold (95% CI 1.2, 6.8). Low folate is a risk factor for MI, particularly in women with the *MTHFR* 677TT genotype. Homocysteine levels were not influenced by OC use.

Keywords: folate, homocysteine, methylenetetrahydrofolate reductase (*MTHFR*) gene, myocardial infarction.

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Introduction

The most common enzyme defect associated with moderate hyperhomocysteinemia is a point mutation, C→T substitution at nucleotide 677 (677C→T), in the coding region of the gene for methylenetetrahydrofolate reductase (*MTHFR*), resulting in a thermolabile *MTHFR* variant with about 50% residual enzyme activity [1]. Among homogeneous populations positive associations were found between homozygous *MTHFR* genotype and cardiovascular disease [2–4], but the *MTHFR* 677TT mutation did not increase cardiovascular risk significantly in the meta-analysis from Brattström [5]. Surprisingly, a negative association was found between the homozygous genotype and cardiovascular disease in postmenopausal women [6].

Elevated plasma homocysteine levels have been associated with a modestly increased risk of cardiovascular disease [7–9], and may be a predictor of mortality in patients with coronary artery disease [10,11]. The latter is suggestive of a prothrombotic effect of hyperhomocysteinemia, which was also found among patients with venous thrombosis [12]. The association between first myocardial infarction (MI) in young women and hyperhomocysteinemia, low folate or vitamin B_{12} and *MTHFR* mutation is less clear.

Data on the effect of female hormones on homocysteine levels are limited to reports on hormone replacement therapy without clinical outcome events [13,14], but studies in young women are sparse. Earlier studies that examined the risk of MI

in relation to hyperhomocysteinemia [7] and *MTHFR* mutation [15] in women did not report on the effect of oral contraceptive (OC) use on homocysteine levels.

In this study we investigated whether the *MTHFR* 677TT genotype, hyperhomocysteinemia, low folate or vitamin B_{12} levels are risk factors for MI in young women. In addition, we compared homocysteine levels in healthy OC users, hormone replacement therapy users and non-users.

Patients and methods

Study design

The Risk of Arterial Thrombosis In relation to Oral contraceptive use (RATIO) study is a nationwide population-based case–control study of the association between OC use and MI. Details of the study have been described before [16]. The study protocol was approved by the ethics committees of the participating hospitals and informed consent was obtained from all participants.

Subjects

We included consecutive patients 18–49 years of age who were hospitalized with a first MI to one of the 16 participating centers (see Appendix) in the Netherlands between January 1990 and October 1995. The patients were selected through a search of the hospital database for acute MI. Medical records and discharge letters were reviewed for confirmation of the diagnostic criteria for MI, which was defined by the presence of symptoms, elevated cardiac enzyme levels, and electrocardiographic changes indicative of MI. Healthy control women were drawn from the general population, by means of random digit dialing in the same geographic areas from which the patients originated. Control women were stratified for age (5 years categories) and index year of MI. There were two phases of data collection. In the first phase, 248 patients and 925 control women filled out a standardized postal questionnaire concerning classical risk factors for MI. In the second phase of the study blood samples were drawn or buccal swabs collected for DNA analysis of *MTHFR* genotypes. Samples of venous blood (203 patients, 638 controls) or buccal swabs (15 patients, 126 controls) were obtained from 218 (88%) patients and 764 (83%) controls for DNA analysis. We asked the women about their use of medication and vitamin supplements such as folic acid, vitamin B_{12}, and vitamin B_6. After excluding vitamin users (21 patients, 13 controls), two control women with severe hyperhomocysteinemia ($>100\,\mu mol\,L^{-1}$), and subjects (one patient, 22 controls) for whom not all measurements could be performed, plasma samples for both homocysteine, folate, and vitamin B_{12} determinations were available in 181 patients and 601 control women.

Blood collection and laboratory analyses

Non-fasting blood samples were collected with a mean interval of 5 years after the index date and drawn from the antecubital vein. Biopool® Stabilyte™ tubes (Biopool, Umea, Sweden), containing $0.5\,mol\,L^{-1}$ acidic citrate for homocysteine measurement, were immediately placed on ice and centrifugated within 4 h. Blood samples were centrifuged at $1440\,g$ for 15 min. The plasma was separated and stored at $-70\,^{\circ}C$ until analysis. Plasma total homocysteine concentration was determined by automated high-performance liquid chromatography in the Laboratory of Pediatrics and Neurology of the University Medical Center Nijmegen after derivatization with monobromobimane as described previously [17]. The intra-assay coefficient of variation was <3.3% and the interassay coefficient was <5%. EDTA blood was collected for vitamin measurements. Folate and vitamin B_{12} concentrations were simultaneously measured using a Dualcount® Radioassay (Diagnostic Product Corp., Los Angeles, CA, USA). Serum creatinine was measured on a clinical analyzer (Roche/Hitachi® 747, Roche Diagnostics, Mannheim, Germany). DNA was obtained by means of venous blood samples or buccal swabs. The *MTHFR* 677C → T mutation was investigated by polymerase chain reaction using the primers used described by Frosst *et al*. [1]

Major cardiovascular risk factors

Subjects were categorized as current smokers when they reported smoking in the year before the index date, or as non-smokers. Women were considered as hypertensive, hypercholesterolemic or diabetic at the time of MI or the index date in control women when they reported a physician's diagnosis or were taking medication for these conditions. Body mass index (BMI) was calculated as body weight (kg) divided by height squared (m^2). A family history of cardiovascular disease was defined as the presence of MI, stroke or peripheral arterial disease under 60 years of age in first-degree relatives. Current OC use was defined as using OCs in the month prior to MI for patients and analogously in the month before the index date for controls.

Statistical analysis

Univariate odds ratios (ORs) were calculated for the association between the *MTHFR* genotypes and MI by logistic regression analysis with 95% confidence intervals (95% CI) as a measure of relative risk. Homocysteine, folate and vitamin B_{12} concentrations of both cases and controls were stratified into quartiles, based on the distribution of these compounds among control women. We calculated the ORs for MI for the three higher levels relative to the lowest reference level for homocysteine and for the three lower levels relative to the highest reference level for folate and vitamin B_{12} [18]. We also investigated a possible dose–response relation for homocysteine by calculating ORs for homocysteine concentrations above 90, 95, and 97.5 percentiles compared with below this cut-off level in a logistic model. In multivariate analyses we adjusted for the stratification variables (age, index year, and area of residence) and putative confounders. ORs were calculated for the *MTHFR* genotypes according to folate status, defined as above or below the median folate level in the control group, and compared with the reference group (CC genotype and folate $\geq 7.4\,nmol\,L^{-1}$).

Table 1 Clinical and laboratory characteristics of women with first myocardial infarction (MI) and control women

Characteristic	MI patients ($n = 181$)	Control women ($n = 601$)	P-value*
Age (years)	42.9 ± 6.0	38.8 ± 7.9	<0.001
History of hypertension (%)†	51 (28)	37 (6)	<0.01
History of hypercholesterolemia (%)†	20 (11)	19 (3)	<0.01
History of diabetes (%)†	8 (4)	9 (1.5)	<0.05
Body mass index, kg/m^2	25.8 ± 5.0	23.4 ± 3.7	<0.001
Cigarette smoking (%)†	147 (81)	259 (43)	<0.01
Family history of cardiovascular disease (%)†	115 (65)	211 (37)	<0.01
Oral contraceptive use (%)†	68 (38)	206 (35)	ns
Systolic/diastolic blood pressure (mmHg)	135.9/85.4	129.3/82.0	<0.01
Creatinine (μmol L^{-1})	76 ± 12	76 ± 11	ns
Homocysteine (μmol L^{-1})	12.7 ± 4.1	12.3 ± 3.4	ns
Folate (nmol L^{-1})	7.3 ± 4.2	8.5 ± 4.3	<0.01
Vitamin B_{12} (pmol L^{-1})	405 ± 191	383 ± 183	ns

Plus-minus values are means ± SD. *Analysis of variance was used to compare differences between means and a χ^2 test was used to compare dichotomous variables. †Data on oral contraceptive use were missing in two patients and eight controls, on family history of cardiovascular disease in five patients and 37 controls, on smoking in four controls, on hypercholesterolemia in three controls and on hypertension and diabetes in two controls.

Results

Descriptive characteristics

Clinical and laboratory characteristics of the patients and control women are shown in Table 1. The mean age of patients was 42.9 (range 24–49) years compared with 38.8 years (range 18–49) in control women. Control women had lower frequencies of classical risk factors for MI, including smoking, hypertension, hypercholesterolemia, diabetes, and family history of cardiovascular disease. Mean homocysteine, creatinine and vitamin B_{12} levels in patients did not differ from control women: homocysteine 12.7 μmol L^{-1} in patients vs. 12.3 μmol L^{-1} in controls, mean difference 0.4 μmol L^{-1} (95% CI 0.2, 1.0); creatinine 76 μmol L^{-1} in patients and 76 μmol L^{-1} in controls; vitamin B_{12} levels 405 pmol L^{-1} in patients vs. 383 pmol L^{-1} in controls, mean difference 23.7 pmol L^{-1} (95% CI −6.9, 54.3); while folate levels were significantly lower in patients than in controls: 7.3 nmol L^{-1} vs. 8.5 nmol L^{-1}, mean difference 1.2 nmol L^{-1} (95% CI 0.5, 1.9).

Table 2 Odds ratios for myocardial infarction in relation with *MTHFR* genotypes

MTHFR genotype	Patients ($n = 181$) N (%)	Control women ($n = 601$) N (%)	Odds ratio (95% CI)
CC	78 (43)	280 (47)	1*
CT	81 (45)	262 (44)	1.1 (0.8, 1.6)
TT	22 (12)	59 (10)	1.3 (0.8, 2.3)

*Reference category. CC, Wild-type genotype; CT, heterozygote genotype; TT, homozygote genotype.

Table 3 Homocysteine, folate levels and vitamin B_{12} levels [mean (SD)] among control women according to *MTHFR* genotypes

MTHFR genotype	Homocysteine (SD)*, μmol L^{-1}	Folate (SD)*, nmol L^{-1}	Vitamin B_{12} (SD), pmol L^{-1}
CC	11.6 (2.7)	8.9 (4.5)	391 (192)
CT	12.4 (3.2)	8.3 (4.1)	373 (173)
TT	14.9 (5.3)	7.1 (4.1)	388 (185)

CC, Wild-type genotype; CT, heterozygote genotype; TT, homozygote genotype. *P for trend <0.01.

MTHFR *genotypes and homocysteine, folate and vitamin B_{12} levels*

The homozygote mutant allele (TT) was detected in 12% of the patients with MI and in 10% of controls (Table 2). The OR for MI for the homozygote TT genotype was 1.3 (95% CI 0.8, 2.3) compared with the CC wild type. The OR for the combination of the homozygote and heterozygote genotypes vs. the wild type was 1.2 (95% CI 0.9, 1.6).

Table 3 shows a gradual increase in mean plasma homocysteine levels according to the *MTHFR* genotypes in control women, with a difference in the mean homocysteine concentration of 3.3 μmol L^{-1} between the wild-type CC and the mutant TT genotype ($P < 0.001$). An inverse association was found for folate levels with a difference of 1.8 nmol L^{-1} between CC and TT genotype, while no association was apparent for vitamin B_{12} levels and these genotypes.

The ORs for MI adjusted for the stratification variables (age, area of residence and index year) were not significantly increased for the highest homocysteine quartiles compared with the reference category (Table 4). We found a positive association of homocysteine levels with hypertension and smoking (both $P < 0.01$) and with creatinine levels ($P = 0.01$). In the case group mean homocysteine was 13.0 μmol L^{-1} in smokers vs. 11.3 μmol L^{-1} in non-smokers (95% CI for difference −3.23, −0.16). In the control group mean homocysteine was 12.8 μmol L^{-1} in smokers vs. 11.9 μmol L^{-1} in non-smokers (95% CI for difference −1.47, −0.38). Folate levels were not significantly different between smokers and non-smokers.

Table 4 Odds ratios for myocardial infarction associated with increasing quartiles of homocysteine, and decreasing quartiles of folate and vitamin B_{12}

	I	II	III	IV
Homocysteine, μmol L^{-1}	<10.10	10.10–11.69	11.70–13.60	>13.60
Cases/controls	37/149	56/160	31/137	57/155
OR (95% CI)*	1†	1.3 (0.8, 2.2)	0.8 (0.5, 1.5)	1.3 (0.8, 2.0)
Folate, nmol L^{-1}	>10.40	7.40–10.40	5.40–7.39	<5.40
Cases/controls	25/150	45/155	53/151	58/145
OR (95% CI)*	1†	2.0 (1.1, 3.4)	2.2 (1.3, 3.8)	3.0 (1.7, 5.1)
Vitamin B_{12}, pmol L^{-1}	>475	350–475	254–349	<254
Cases/controls	47/145	51/154	43/152	40/150
OR (95% CI)*	1†	1.0 (0.6, 1.7)	1.0 (0.6, 1.6)	1.1 (0.6, 1.7)

Quartiles based on the distribution among control women. *Odds ratios adjusted for the stratification variables (age, index year and area of residence). †Reference category.

Table 5 Odds ratios (95% CI)* for myocardial infarction by strata of *MTHFR* 677C→T variant and plasma folate status

	MTHFR 677C→T		
	CC	CT	TT
Patients/control women (*n*)	78/280	81/262	22/59
High folate status	1†	0.9 (0.5, 1.6)	0.6 (0.2, 2.3)
Low folate status	1.3 (0.8, 2.2)	1.6 (1.0, 2.6)‡	2.0 (1.0, 3.7)‡

Folate status was defined as above or below the median folate level (7.4 nmol L^{-1}) in control women. *Adjusted for stratification variables (age, index year and area of residence). †Reference category. ‡$P \leq 0.05$.

At the 90, 95 and 97.5 percentiles (cut-off points 16.1 μmol L^{-1}, 18.6 μmol L^{-1} and 21.0 μmol L^{-1}), the adjusted ORs for higher vs. lower values of homocysteine levels were 1.4 (95% CI 0.9, 2.3); 1.8 (95% CI 0.9, 3.5) and 2.8 (95% CI 1.2, 6.8), respectively, indicating a graded dose–effect relationship. Additional adjustment for currently smoking cigarettes, hypertension, and creatinine attenuated the ORs for hyperhomocysteinemia to 1.2, 1.4 and 1.8, respectively (all ns).

The adjusted ORs for MI increased with decreasing quartiles of folate levels, the OR for the lowest quartile of folate levels <5.4 nmol L^{-1} was 3.0 (95% CI 1.7, 5.1). Further adjustment for putative confounders (smoking, hypertension, diabetes, hypercholesterolemia and BMI) did not change the OR significantly. There was no association between vitamin B_{12} levels and the risk of MI.

The risk of MI for the *MTHFR* genotypes stratified for high and low folate levels according to the median value is shown in Table 5. Patients with low folate status and the CT heterozygote genotype had a 1.6 (95% CI 1.0, 2.6)-fold increased risk of MI compared with women with the CC wild type and high folate status. In patients with low folate status and the homozygous TT genotype the OR for MI was 2.0 (95% CI 1.0,3.7)-fold increased compared with carriers of the CC wild type and high folate status. Further adjustment for putative confounders (smoking, hypertension, diabetes, hypercholesterolemia and BMI) did not change the ORs materially. The results show that the TT genotype is associated with an increased risk of MI only when folate status is low.

Effect of exogenous female hormones on homocysteine and vitamin levels in healthy controls

Mean homocysteine levels and folate levels did not differ between control women who used OCs and those who did not (Table 6). However, mean vitamin B_{12} levels were significant lower in OC users compared with non-users. Among the small group of hormone replacement therapy users (48 controls) we saw no different values of homocysteine and folate from those in non-users.

Discussion

In this population-based case–control study the *MTHFR* 677TT mutation was associated with elevated plasma homocysteine

Table 6 Mean plasma homocysteine, folate and vitamin B_{12} levels in patients and control women according to oral contraceptive use and hormone replacement therapy*

	Oral contraceptive use		No oral contraceptive use		Hormone replacement therapy	
	Patients $n = 24$	Controls $n = 128$	Patients $n = 133$	Controls $n = 419$	Patients $n = 22$	Controls $n = 48$
Homocysteine (SD), μmol L^{-1}	11.6 (2.5)	12.2 (3.2)	12.9 (4.4)	12.3 (3.5)	12.9 (4.1)	12.0 (3.1)
Folate (SD), nmol L^{-1}	8.1 (5.1)	7.9 (4.3)	6.9 (3.6)	8.5 (4.3)†	9.0 (6.2)	9.0 (4.1)
Vitamin B_{12} (SD), pmol L^{-1}	322 (135)	293 (159)†‡	421 (200)	411 (188)	404 (171)	378 (120)‡

*Data on oral contraceptive or hormone replacement use at the time of blood collection were missing in two patients and six control women. †$P < 0.001$ for oral contraceptive users vs. no oral contraceptive users within control women. ‡$P = 0.001$ for oral contraceptive users vs. hormone replacement therapy users within control women.

concentrations. Homocysteine increased the risk of MI only significantly at very high levels exceeding the 97.5th percentile in the control group. Low plasma folate levels were associated with a 2–3-fold increased risk of MI compared with the highest quartile, and the effect of low folate status was most pronounced in patients with the TT genotype. Homocysteine levels did not differ between OC users, hormone replacement users and non-users.

Vitamins are important cofactors in the enzymatic pathway of homocysteine metabolism, and earlier studies provided data that plasma folate levels and to a lesser extent plasma vitamin B_{12} were inversely related to plasma homocysteine levels [19]. We confirmed these inverse assocations between homocysteine and folate levels, as well as vitamin B_{12} levels, but did not find an increased risk of MI among women in the lowest quartile of vitamin B_{12} levels. Folate deficiency in young women may precede hyperhomocysteinemia, especially in women with the *MTHFR* 677TT mutation, and therefore low folate may be the most relevant risk factor for MI. This observation was also found in young women from the USA [15].

The frequency of the 677TT genotype was slightly higher in patients compared with controls, in line with the distributions found in different homogeneous populations in Europe [2,3,20]. In the control group of young women we found a strong dose–response relation between the *MTHFR* mutation and homocysteine levels, in accordance with earlier studies [21]. There is debate on the causality of both the *MTHFR* 677TT genotype and hyperhomocysteinemia in their association with MI. The most recent meta-analysis of retrospective and prospective case–control studies, on the risk of cardiovascular disease and the *MTHFR* genotypes, showed heterogeneity in the results between European and American populations, probably explained by a different folate status [22]. The young age of the patients and the absence of previous cardiovascular events could have contributed to the absence of a difference in homocysteine levels between patients and controls. Risk-modifying factors after MI, such as dietary intake and medication, could have influenced homocysteine levels in patients, and this could have theoretically led to an underestimation of homocysteine as a risk factor.

We found no difference in the levels of homocysteine and folate between women who used OCs and those who did not, demonstrating that the increased risk of MI due to OCs is not mediated by homocysteine or folate levels. Increased levels of homocysteine during OC use was reported from one study, which, however, included measurements during one cycle of OC use [23], and which could not be confirmed by others [24]. The lower vitamin B_{12} levels in OC users compared with non-users is in agreement with the literature [25,26]

There are some potential limitations in this study. Our study size was too small to draw definite conclusions on all determinants that were studied. However, this is the largest study performed among young women and the results are consistent with those from other recent investigations. Even in the largest meta-analysis the *MTHFR* TT genotype was a weak risk factor for coronary heart disease, with an OR of 1.16 (95% CI 1.05, 1.28), and elevated homocysteine levels were at most a modest predictor of cardiovascular disease [9,22]. We cannot exclude completely an effect of including only non-fatal cases. If folate levels or homocysteine levels are related to case fatality, the selection of survivors of MI may have led to a small underestimation of the odds ratios. Folate, vitamin B_{12} and homocysteine levels were measured after the event, and we do not know whether nutritional intake of folate or other lifestyle habits have been changed in patients after MI. In addition, we can not exclude the influence of risk-modifying medication on homocysteine and folate levels. However, the inverse association between folate levels and homocysteine was also clearly prominent in the control group. We did not measure homocysteine levels after oral methionine loading, which may be determined to a greater extent by the transsulfuration pathway in which vitamin B_6 is a cofactor [27]. Neither did we assess pyridoxine levels; although a few studies showed a protective effect of higher pyridoxine levels on coronary heart disease [28,29], in most studies pyridoxine has not been proven an important risk factor for MI. Homocysteine levels were measured under non-fasting conditions. However, it is unlikely that the risk estimates in this study would be influenced by the blood sampling procedure, which was equal for patients and controls.

In the present study we found an increased risk of MI for low plasma folate concentrations in accordance with the study in young women from Schwartz [15]. As hyperhomocysteinemia is easily corrected by vitamin supplementation as well as folic acid fortification of meals, and vitamin supplementation in subjects with normal vitamin levels lead to further decreases of homocysteine levels [30], this offers an important perspective for prevention of premature cardiovascular disease [31,32]. Recently, beneficial effects of folic acid have been reported which are largely independent of homocysteine lowering [33]. It seems reasonable await data from randomized controlled trials before implementing active screening or treatment programs, although treatment of low vitamin levels may be defensible today.

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