

Variations in folate pathway genes are associated with unexplained female infertility

Signe Altmäe, M.Sc.,^{a,b} Anneli Stavreus-Evers, Ph.D.,^c Jonatan R. Ruiz, Ph.D.,^d Margit Laanpere, M.Sc.,^b Tiina Syvänen, M.Sc.,^c Agneta Yngve, Ph.D.,^e Andres Salumets, Ph.D.,^{b,f} and Torbjörn K. Nilsson, M.D., Ph.D.^g

^a Department of Clinical Science, Intervention and Technology, Division of Obstetrics and Gynecology, Karolinska Institutet, Karolinska University Hospital Huddinge, Stockholm, Sweden; ^b Department of Biotechnology, Institute of Molecular and Cell Biology, University of Tartu, Tartu, Estonia; ^c Department of Women's and Children's Health, Uppsala University, Akademiska Sjukhuset, Uppsala; ^d Department of Biosciences and Nutrition, Unit for Preventive Nutrition; and ^e Department of Biosciences and Nutrition, Unit for Public Health Nutrition, Karolinska Institutet, Stockholm, Sweden; ^f Department of Obstetrics and Gynecology, University of Tartu, Tartu, Estonia; and ^g Department of Clinical Chemistry, Örebro University Hospital, Örebro, Sweden

Objective: To investigate associations between folate-metabolizing gene variations, folate status, and unexplained female infertility.

Design: An association study.

Setting: Hospital-based IVF unit and university-affiliated reproductive research laboratories.

Patient(s): Seventy-one female patients with unexplained infertility.

Intervention(s): Blood samples for polymorphism genotyping and homocysteine, vitamin B12, and folate measurements.

Main Outcome Measure(s): Allele and genotype frequencies of the following polymorphisms: 5,10-methylenetetrahydrofolate reductase (*MTHFR*) 677C/T, 1298A/C, and 1793G/A, folate receptor 1 (*FOLR1*) 1314G/A, 1816delC, 1841G/A, and 1928C/T, transcobalamin II (*TCN2*) 776C/G, cystathionase (*CTH*) 1208G/T and solute carrier family 19, member 1 (*SLC19A1*) 80G/A, and concentrations of plasma homocysteine, vitamin B12, and serum folate.

Result(s): *MTHFR* genotypes 677CT and 1793GA, as well as 1793 allele A were significantly more frequent among controls than in patients. The common *MTHFR* wild-type haplotype (677, 1298, 1793) CAG was less prevalent, whereas the rare haplotype CCA was more frequent in the general population than among infertility patients. The frequency of *SLC19A1* 80G/A genotypes differed significantly between controls and patients and the A allele was more common in the general population than in infertile women. Plasma homocysteine concentrations were influenced by *CTH* 1208G/T polymorphism among infertile women.

Conclusion(s): Polymorphisms in folate pathway genes could be one reason for fertility complications in some women with unexplained infertility. (Fertil Steril® 2009; ■:■-■. ©2009 by American Society for Reproductive Medicine.)

Key Words: Female infertility, homocysteine, *MTHFR*, *FOLR1*, *TCN2*, *CTH*, *SLC19A1*

Folate is an important B vitamin that is believed to be crucial for reproduction (1). Folate metabolism is involved in a large number of physiological and pathophysiological processes in the

Received December 15, 2008; revised January 29, 2009; accepted February 9, 2009.

S.A. has nothing to disclose. A.S.-E. has nothing to disclose. J.R.R. has nothing to disclose. M.L. has nothing to disclose. T.S. has nothing to disclose. A.Y. has nothing to disclose. A.S. has nothing to disclose. T.K.N. has nothing to disclose.

Supported by the European Union via the European Regional Development Fund and the Centre of Excellence in Genomics, Estonian Biocentre and Tartu University; the Estonian Ministry of Education and Science (core grants nos. 0182641s04 and PBGMR07903); the Estonian Science Foundation (grant no. 6498); the Spanish Ministry of Education (EX-2007-1124); The Swedish Research Council (2005-7293); the Swedish Society of Medicine; the Magn. Bergvalls Foundation; the Goljes Foundation; the Åke Wibergs Foundation; Nyckelfonden, Örebro; Karolinska Institutet, and the County Council of Stockholm and Örebro.

Reprint requests: Signe Altmäe, M.Sc., Department of Obstetrics and Gynecology K57, Karolinska Institutet, Karolinska University Hospital Huddinge, 14186 Stockholm, Sweden (FAX: +46858587575; E-mail: signe.altmae@ki.se).

body. Folates participate in amino acid metabolism, purine and pyrimidine synthesis, and methylation of nucleic acids, proteins, and lipids. Dietary or genetically determined folate deficiency may impair the function of these metabolic pathways and lead to homocysteine accumulation (2). Homocysteine, a thiol-containing amino acid, originates from the one-carbon-donating metabolism of methionine and is remethylated to methionine, with folates acting as methyl donors (3).

Possible unfavorable effects of folate deficiency and homocysteine accumulation on female reproductive functions include reduced cell division, inflammatory cytokine production (4), altered nitric oxide metabolism (5), increased oxidative stress (6), elevated apoptosis (7), and disturbed methylation reactions (8). All of these processes are involved in oocyte development, preparation of the endometrial receptivity, embryo implantation, and also, in the following pregnancy.

Severe maternal folate deficiency before conception and during gestation has been shown to hamper female fertility

and fetal viability in several animal models, emphasizing the essentiality of folate during mammalian folliculogenesis and fetal development (9). In humans, preconception folic acid supplementation has been shown to increase folate levels and decrease those of homocysteine in follicular fluid (10). In addition, regular use of multivitamin supplements including folate has recently been reported to decrease the risk of anovulatory infertility (11). Furthermore, periconceptional supplementation with folate and vitamin B12 has been found to be associated with a lower incidence of miscarriage in women planning pregnancy (12).

Several variations have been identified in genes involved in folate absorption and folate-mediated one-carbon metabolism. These polymorphisms may alter the beneficial effect of folates and other B vitamins that play a role in the metabolism of methyl groups and change the flux of folate cofactors between DNA synthesis and methylation reactions (13). The most important variation in folate metabolism in terms of prevalence and impact seems to be the 5,10-methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphism 677C/T (14). The *MTHFR* gene is involved in the folate methylation cycle, where homocysteine is converted to methionine. Methionine is the precursor of the methyl group donor *S*-adenosylmethionine (SAM), which is used in the methylation of DNA, proteins, and lipids (15). The *MTHFR* 677C/T variation results in an amino acid change at codon Ala222Val, giving rise to an unstable enzyme with reduced activity (14). This polymorphism results in the accumulation of homocysteine (16) and impaired methylation reactions. Methylation of DNA is one of the most common repressor mechanisms of tissue-specific genes. Thus, inefficient methylation caused by this polymorphism may affect gene regulation.

These findings lead us to the question of what influence, if any, do polymorphisms in folate pathway-related genes have on female fertility and on folate status among women with unexplained infertility. More than 10% of infertile couples suffer from infertility of an unexplained nature (17). The women in these couples have normal ovulatory cycles and hormonal profiles, and no organ pathologies. Their partners show no evidence of semen quality problems. Hence, the women may be unable to conceive as a result of disturbances in oocyte quality or in endometrial maturation resulting from impaired folate metabolism. To validate this hypothesis we studied the prevalence of 10 polymorphisms in genes involved in the folate pathway (*MTHFR* 677C/T, *MTHFR* 1298A/C, *MTHFR* 1793G/A, folate receptor 1 (*FOLR1*) 1314G/A, *FOLR1* 1816delC, *FOLR1* 1841G/A, *FOLR1* 1928C/T, transcobalamin II (*TCN2*) 776C/G, cystathionase (*CTH*) 1208G/T and solute carrier family 19, member 1 (*SLC19A1*) 80G/A) among women with unexplained infertility and in controls from the general population. In addition, we evaluated the effects of these 10 polymorphisms on blood folate, vitamin B12, and homocysteine concentrations among infertile women.

MATERIALS AND METHODS

Subjects

The Ethics Committee of Karolinska Institutet approved the study and informed consent was obtained from participating women. The patient group consisted of 71 women with unexplained infertility who attended the Department of Obstetrics and Gynecology, Karolinska University Hospital Huddinge, from 2000–2007. All of the women were of Swedish or Finnish origin. Unexplained infertility was diagnosed after the couple had undergone a standard set of diagnostics procedures and tests that also included hormone assays and there had been at least two analyses of the partner's semen, showing normal results according to World Health Organization criteria (18). The mean age of the women was 33.1 ± 3.2 (SD) years, mean body mass index (BMI) was 21.7 ± 2.5 kg/m², mean cycle length was 28.4 ± 1.8 days, and the mean duration of menses was 4.7 ± 0.8 days. All women had normal ovarian function. Their serum concentration of FSH was not more than 11 IU/L during the early follicular phase of cycle days 2–5. All women had a serum PRL concentration <20 µg/L and normal TSH as well as thyroid hormone serum levels. In addition, all women showed normal tubal patency in hysterosonosalpingography, and no recognizable endometriosis according to symptoms, clinical examination, ultrasonography, or diagnostic laparoscopy. However, according to our internal guidelines, only the women with suspicion of endometriosis underwent diagnostic laparoscopic examination. The control group consisted of 1,079 individuals from a cross-sectional population studied in central Sweden, the same area in which our group of infertile women was recruited. The data concerning the control individuals have already been published (19–22).

Homocysteine, Folate, and Vitamin B12 Assays

Homocysteine was assayed in acidified citrate plasma using a fluorescence polarization immunoassay and an IMx unit (Abbott Laboratories, Chicago, IL). Concentrations of serum folate were measured by means of a solid-phase time-resolved fluoroimmunoassay based on a competitive reaction between europium-labeled pteroyl-glutamic acid, the stable form of folate, and sample folate for a limited number of binding sites on folate-binding protein (AutoDelfia Folate; Wallac Oy, Turku, Finland). Plasma vitamin B12 concentrations were measured by means of a fluorometric method with an Abbott IMx autoanalyzer (Abbott Laboratories). All coefficients of variation were $<7.5\%$.

Genotyping

Genomic DNA for polymorphism analysis was extracted from EDTA-collected peripheral blood using a QIAamp DNA Blood Maxi kit (Qiagen, Venlo, the Netherlands). Previously described Pyrosequencing assays (19–22) (Biotage AB, Uppsala, Sweden) were used to genotype the polymorphisms *MTHFR* 677C/T (rs1801133), *MTHFR* 1298A/C (rs1801131), *MTHFR* 1793G/A (rs2274976), *FOLR1*

1314G/A (rs2071010), *FOLR1* 1816delC (rs3833748), *FOLR1* 1841G/A (rs1540087), *FOLR1* 1928C/T (rs9282688), *TCN2* 776C/G (rs1801198), *CTH* 1208G/T (rs1021737), and *SLC19A1* 80G/A (rs1051266).

Statistical Analysis

All analyses were performed using Statistical Package for Social Sciences statistical software (SPSS v. 16.0 for Macintosh; SPSS Inc., Chicago, IL), with the exception of haplotype analyses, which were performed with Haploview software (version 4.1) (23). Data are given as mean \pm SD, unless otherwise indicated. Nominal variables were analyzed by χ^2 tests. Allele frequencies were calculated to investigate deviation from Hardy-Weinberg equilibrium. All continuous variables were normally distributed, except for serum folate concentrations, which were logarithmic transformed. We analyzed the influence of polymorphisms on folate and vitamin B12 concentrations in infertile women by one-way analysis of variance (ANOVA), whereas mean concentrations of folate and vitamin B12 in genotype subgroups were compared by using Tukey's test. The effects of polymorphisms and *MTHFR* haplotypes on plasma homocysteine concentrations among infertile women were calculated by using analysis of covariance (ANCOVA) after adjusting for folate and age. Polymorphisms and haplotypes were entered as fixed factors and homocysteine as a dependent variable. In calculations of covariance, Bonferroni correction was used. For all analyses, a *P* value $<$.05 was considered statistically significant.

RESULTS

Allele and Genotype Frequencies

The genotype and allele frequencies of polymorphisms *MTHFR* 677C/T, *MTHFR* 1298A/C, *MTHFR* 1793G/A, *FOLR1* 1314G/A, *FOLR1* 1816delC, *FOLR1* 1841G/A, *FOLR1* 1928C/T, *TCN2* 776C/G, *CTH* 1208G/T, and *SLC19A1* 80G/A are presented in Table 1. Data from women with unexplained infertility were compared with data from cross-sectional population studies conducted in the same region (19–22). All genotype distributions in the study subjects were in Hardy-Weinberg equilibrium. Significant differences in allele frequencies between controls and infertile women were detected in polymorphisms *MTHFR* 1793G/A, with G allele prevalences of 95.3% and 99.2% (*P* = .026) and in *SLC19A1* 80G/A, with G allele frequencies of 55.8% and 59.7% (*P* = .002). A significant difference in genotype distribution between the study groups was seen in *SLC19A1* 80G/A (*P* = .011), where the GG genotype was represented in 32.9% of the controls and 35.7% of the infertile women, the GA genotype in 45.8% and 48.2%, and the AA genotype in 21.3% and 16.1%, respectively. The frequencies of variant heterozygous and homozygous genotypes of the studied polymorphisms are shown in Figure 1. Significant differences in the frequencies of heterozygous genotypes between controls and infertile women were detected with regard to polymorphisms *MTHFR* 677C/T (43.6% and 32.4%, respectively; *P* = .043) and *MTHFR* 1793G/A (9.1% and 1.4%, respec-

tively; *P* = .012). A significant difference in the frequencies of homozygous variant genotypes between study groups was seen with polymorphism *SLC19A1* 80G/A; AA genotype frequencies being 21.3% and 16.1% (*P* = .026) in controls and infertile women.

Haplotype Analysis

Haplotype analysis of two (677, 1298) and three (677, 1298, 1793) *MTHFR* loci revealed three and four haplotypes, respectively. The prevalence of *MTHFR* haplotypes in controls and women with unexplained infertility is presented in Table 2. The *MTHFR* 677-1298 CA nonmutated haplotype occurred significantly less frequently among control subjects when compared with infertile women (36.4% vs. 45.8%; *P* = .028). The same pattern was seen when the data were analyzed as a three-locus system—the nonmutated *MTHFR* 677-1298-1793 haplotype CAG was less frequent among controls than in infertile women (36.4% vs. 45.8%, respectively; *P* = .028). The *MTHFR* haplotype CCA was detected more frequently in controls than in infertile women (4.7% vs. 0.7%, respectively; *P* = .026).

Blood Homocysteine, Folate, and B12 Concentrations in Infertile Women

Among infertile women, whose serum and plasma samples were stored, the mean concentrations of serum folate, plasma vitamin B12, and homocysteine were well within the reference intervals: 19.2 ± 14.0 nmol/L (*n* = 66), 332.5 ± 106.9 pmol/L (*n* = 28), and 8.2 ± 2.7 μ mol/L (*n* = 44), respectively. A total of 83.0% of the infertile women were using folate supplements during the study. Analysis of variance did not show any correlation between the studied polymorphisms and serum folate and plasma B12 concentrations (data not shown). Analysis of covariance was used to assess the effects of the studied polymorphisms and haplotypes of the *MTHFR* gene on homocysteine concentrations among women with unexplained infertility, as folate, vitamin B12, and age are cofactors and biological covariates in the metabolism of homocysteine. However, no effect of vitamin B12 on homocysteine levels was detected; therefore B12 was excluded from further covariance analyses. Serum folate concentrations were negatively correlated with homocysteine values (*P* $<$.05). Homocysteine concentrations in relation to all studied polymorphisms are shown in Table 3. The TT genotype of the *CTH* 1208G/T polymorphism had an increasing effect on homocysteine levels among infertile women (*P* = .033), regardless of all these women were taking folate supplements. Analysis of covariance did not reveal any effect of *MTHFR* haplotypes on homocysteine concentrations in the women with unexplained infertility (data not shown).

DISCUSSION

Our findings indicate that polymorphisms *MTHFR* 677C/T and 1793G/A, as well as *SLC19A1* 80G/A, may account for infertility in women with an otherwise unspecified reason

TABLE 1

Genotype and allele frequencies of polymorphisms in genes of the folate-metabolizing pathway in controls from a Swedish population and women with unexplained infertility.

		Controls	χ^2	Infertile women	χ^2	P value
MTHFR 677C/T ⁽²⁸⁾	CC	330 (47.7)	0.605	40 (56.3)	2.481	.183
	CT	302 (43.6)		23 (32.4)		
	TT	60 (8.7)		8 (11.3)		
	p(C)	0.685		0.725		
	q(T)	0.305		0.275		
MTHFR 1298A/C ⁽²⁸⁾	AA	302 (43.6)	1.785	39 (54.9)	0.307	.187
	AC	322 (46.5)		26 (36.6)		
	CC	68 (9.8)		6 (8.5)		
	p(A)	0.669		0.740		
	q(C)	0.331		0.291		
MTHFR 1793G/A ⁽²⁸⁾	GG	628 (90.8)	0.200	70 (98.6)	0.003	.079
	GA	63 (9.1)		1 (1.4)		
	AA	1 (0.1)		0		
	p(G)	0.953		0.992		
	q(A)	0.047		0.008		
FOLR1 1314G/A ⁽²⁷⁾	GG	338 (86.9)	0.024	57 (87.7)	0.279	.843
	GA	49 (12.6)		8 (12.3)		
	AA	2 (0.5)		0		
	p(G)	0.932		0.936		
	q(A)	0.068		0.064		
FOLR1 1816delC ⁽²⁷⁾	CC	387 (99.5)	0.0026	54 (96.4)	0.018	.079
	Cdel	2 (0.5)		2 (3.6)		
	DelDel	0		0		
	p(C)	0.997		0.981		
	q(-)	0.003		0.019		
FOLR1 1841G/A ⁽²⁷⁾	GG	386 (99.5)	0.0026	54 (96.4)	0.018	.079
	GA	2 (0.5)		2 (3.6)		
	AA	0		0		
	p(G)	0.997		0.981		
	q(A)	0.003		0.019		
FOLR1 1928C/T ⁽²⁷⁾	CC	368 (95.1)	0.245	54 (96.4)	0.018	1.000
	CT	19 (4.9)		2 (3.6)		
	TT	0		0		
	p(C)	0.975		0.981		
	q(T)	0.024		0.019		
TCN2776C/G ⁽²⁹⁾	CC	124 (31.9)	0.695	20 (32.2)	0.170	.997
	CG	184 (47.3)		29 (46.8)		
	GG	81 (20.8)		13 (21)		
	p(C)	0.555		0.568		
	q(G)	0.445		0.458		
CTH 1208G/T ^a	GG	203 (52.2)	1.521	22 (39.3)	0.504	.191
	GT	156 (40.1)		29 (51.8)		
	TT	30 (7.7)		5 (8.9)		
	p(G)	0.723		0.627		
	q(T)	0.277		0.298		
SLC19A1 80G/A ⁽³⁰⁾	GG	128 (32.9)	2.005	20 (35.7)	0.0004	.011
	GA	178 (45.8)		27 (48.2)		
	AA	83 (21.3)		9 (16.1)		
	p(G)	0.558		0.597		
	q(A)	0.442		0.401		

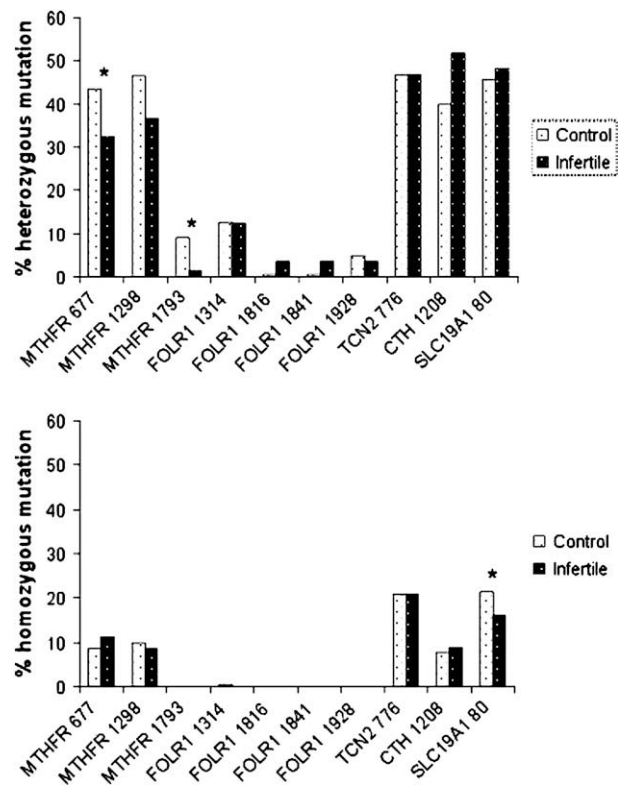
Note: The numbers of subjects and percentages are shown, and χ^2 in Hardy-Weinberg equilibrium testing. Values of *P* indicate the significance of differences in genotype and allele frequencies between the study groups.

^a Unpublished data, T.K. Nilsson 2008.

Altmäe. Variations in folate pathway genes. Fertil Steril 2009.

FIGURE 1

Percentages of subjects heterozygous and homozygous for polymorphisms in folate-metabolizing pathway genes among controls from a Swedish population in comparison with women with unexplained infertility. Heterozygosity was compared with both homozygous genotypes. Minor allele homozygosity was compared with heterozygous and wild-type homozygous genotypes. *Statistically significant difference in genotype frequencies between study groups ($P < .05$).



Altmäe. Variations in folate pathway genes. *Fertil Steril* 2009.

for their infertility. To the best of our knowledge this is the first time that an association between multiple polymorphisms in folate-metabolizing genes and unexplained female infertility has been shown. Keeping in mind the interrelationship between low folate status and elevated blood homocysteine levels, it is important in the context of female infertility to understand the genetic background factors influencing the balance between these two essential compounds. Knowledge of such factors could facilitate prompt identification and treatment of those women trying to achieve pregnancy but who have an unfavorable genetic background and an augmented risk of folate metabolism abnormalities.

Both folate deficiency and hyperhomocysteinemia are known risk factors of pregnancy complications (1). In folliculogenesis, hyperhomocysteinemia may activate apoptosis,

TABLE 2

Haplotype prevalences of *MTHFR* 677C/T, 1298A/C, and 1793G/A polymorphisms in controls and in women with unexplained infertility.

Haplotype	Controls	Infertile	P value
Two-locus system 677-1298			
CA	0.364	0.458	.028
CC	0.331	0.268	.125
TA	0.305	0.275	.454
Three-locus system 677-1298-1793			
CAG	0.364	0.458	.028
TAG	0.305	0.275	.454
CCG	0.284	0.261	.555
CCA	0.047	0.007	.026

Note: Values of P indicate haplotype prevalence differences between the study groups.

Altmäe. Variations in folate pathway genes. *Fertil Steril* 2009.

thereby leading to follicular atresia (8). Negative correlations between follicular fluid homocysteine concentrations and the degree of maturity of retrieved oocytes (24) and in vitro embryo quality on culture day 3 have also been reported (25). However, the results of a recent study have shown a positive correlation between follicular homocysteine concentrations and diameter of the follicle (10). Hyperhomocysteinemia also affects IVF outcome, as pregnancy and implantation rates have been shown to be significantly lower, whereas the abortion rate is higher in women with elevated homocysteine concentrations (26).

It is commonly known that individuals carrying the *MTHFR* 677 T allele, particularly TT homozygotes, have increased plasma homocysteine concentrations. However, people with the 677 TT genotype have increased blood homocysteine concentrations when their folate intake is insufficient, but normal homocysteine values when folate intake is adequate (27). In our study group of infertile women, no effect on plasma homocysteine concentrations was detected in connection with any of the *MTHFR* polymorphisms. Although a haplotype-based approach has been reported to be somewhat superior to a simple genotype-based approach in detecting a genetic influence on homocysteine concentrations (20), no association was found between *MTHFR* haplotypes and homocysteine concentrations. However, it is of importance that the majority of the infertile women had been taking folate supplements, thus the adverse effects of *MTHFR* gene variations might have been masked by sufficient folate intake.

Of the other tested polymorphisms, only *CTH* 1208G/T appeared to influence homocysteine concentrations, irrespective of the folate status and supplement use. Similarly to

TABLE 3

Homocysteine concentrations ($\mu\text{mol/L}$) in relation to polymorphisms in folate-metabolizing pathway genes among women with unexplained infertility.

Genotype (n)	Mean \pm SD	P value
<i>MTHFR</i> 677		
CC (22)	8.19 \pm 0.45	
CT (12)	7.58 \pm 0.61	
TT (8)	9.33 \pm 0.74	.192
<i>MTHFR</i> 1298		
AA (24)	8.59 \pm 0.43	
AC (17)	7.78 \pm 0.52	
CC (1)	7.50 \pm 2.21	.471
<i>MTHFR</i> 1793		
GG (41)	8.29 \pm 0.33	
GA (1)	5.84 \pm 2.11	
AA (0)	—	.257
<i>FOLR1</i> 1314		
GG (35)	8.40 \pm 0.36	
GA (7)	7.42 \pm 0.86	
AA (0)	—	.312
<i>FOLR1</i> 1816		
CC (32)	8.36 \pm 0.39	
Cdel (2)	7.63 \pm 1.62	
deldel (0)	—	.666
<i>FOLR1</i> 1841		
GG (32)	8.36 \pm 0.39	
GA (2)	7.63 \pm 1.62	
AA (0)	—	.666
<i>FOLR1</i> 1928		
CC (33)	8.30 \pm 0.39	
CT (1)	9.16 \pm 2.30	
TT (0)	—	.713
<i>TCN2</i> 776		
CC (14)	8.15 \pm 0.61	
CG (12)	8.56 \pm 0.66	
GG (8)	8.26 \pm 0.83	.896
<i>CTH</i> 1208		
GG (13)	7.88 \pm 0.57	
GT (18)	8.11 \pm 0.48	
TT (3)	11.50 \pm 1.20	.033
<i>SLC19A1</i> 80		
GG (12)	8.92 \pm 0.63	
GA (15)	8.45 \pm 0.56	
AA (7)	7.02 \pm 0.83	.200

Note: Homocysteine concentrations have been adjusted for folate and age.

Altmäe. Variations in folate pathway genes. *Fertil Steril* 2009.

converts cystathionine to cysteine in the *trans*-sulfuration pathway. The *CTH* 1208G/T polymorphism causes a change in the conserved residue Ser403Ile, which might influence enzyme activity and thereby the folate metabolism (28). However, this result should be interpreted with caution as a result of the limited sample size of infertile women with a TT genotype. Further investigation with a larger study group of women with infertility and early pregnancy complications is warranted.

Our finding that the *MTHFR* 677 heterozygous CT genotype was less prevalent among the infertile women than among controls is unexpected, as *MTHFR* 677 T allele carriers have previously been shown to have ovulatory disturbances, diminished responses to ovarian stimulation, and lower serum E₂ concentrations (29, 30). However, in agreement with our result, a recent study revealed that the *MTHFR* 677 CT heterozygous genotype, rather than the homozygous CC genotype, is associated with increased chances of having had a previous IVF pregnancy and a live birth in the current IVF cycle (31). Furthermore, spontaneously aborted embryos have been shown to exhibit a significantly higher frequency of the *MTHFR* 677 CC wild-type genotype and a lower frequency of the heterozygous CT genotype compared with child and adult control groups (32). Correspondingly, the *MTHFR* 677 T allele has been suggested to increase embryo viability in the presence of an adequate folate-containing diet, based on the observation that the T allele frequency has risen in the Spanish population over the years (33). Decreased viability of embryos with the *MTHFR* 677 CC genotype may be caused by increased DNA hypermethylation associated with the more active form of the wild-type MTHFR enzyme, indicating that elevated methionine concentration may have more influence on embryo survival than high homocysteine concentrations (32).

In addition to the lower prevalence of the *MTHFR* 677 CT genotype, we detected the 1793 wild-type G allele more frequently and the GA genotype less frequently in infertile women than in the general population. The *MTHFR* 1793G/A polymorphism results in Arg594Gln amino acid substitution (34). The functional relevance of this variation is not clear, although higher homocysteine concentrations have been reported in association with the wild-type genotype among Swedish adolescents (20). We also detected higher homocysteine values among 1793 GG carriers, but not at a statistically significant level.

The *MTHFR* polymorphisms 677C/T, 1298A/C, and 1793G/A were found to be in linkage disequilibrium among our study group of infertile women, in agreement with previous reports (35, 36). The common wild-type haplotype CAG was more prevalent among infertile women than in the general population. This unexpected result could be explained by the high prevalence of heterozygosity in all *MTHFR* polymorphisms studied among control individuals. As indicated previously, heterozygosity at the *MTHFR* gene locus could possibly be beneficial in terms of effective reproduction.

Along this line of thinking, the *MTHFR* 677C/T polymorphism has been proposed to provide protection against some forms of cancer (37).

Collectively, our findings indicate that variations in the *MTHFR* gene have a role in female infertility. Besides altering homocysteine concentrations, *MTHFR* gene variants have been shown to play role in hemostasis (38, 39). Based on these previous studies it could be hypothesized that polymorphisms in the *MTHFR* gene affect embryo implantation by altering the hemostatic balance between hemorrhage and thrombosis. The hemostatic balance may prove critical at the time of implantation, when the blastocyst interacts with the endometrium and blastocyst-derived syncytiotrophoblasts breach endometrial blood vessels, thereby establishing the primordial uteroplacental circulation. Indeed, inherited thrombophilias are associated with implantation failure (38, 40). Furthermore, the genes encoding thrombogenic proteins are involved, in addition to participating in coagulation processes, in fertilization, embryogenesis, and tissue remodeling (41).

Another important finding in our study is the association between the major allele G of the polymorphism *SLC19A1* 80G/A, as well as wild-type and heterozygous genotypes, and unexplained female infertility. The *SLC19A1* gene encodes the protein reduced folate carrier, which is considered to be the major folate transporter at physiological conditions in most tissues (42). The variation 80G/A introduces the amino acid change His27Arg (43). If the polymorphism were to interfere with the folate-transporting capacity of the reduced folate carrier, alterations in folate concentrations at the site of embryo implantation could have a negative effect on the rapidly dividing embryonic and maternal cells. However, cellular folate intake has been shown not to be affected in vitro by this variation (44). Nonetheless, GG genotype carriers present with elevated plasma homocysteine concentrations (43). Likewise, we detected higher homocysteine concentrations among infertile women carrying the G allele, although that did not reach a statistically significant level. Thus, the negative effect of the *SLC19A1* 80G allele on fertility could be mediated by elevated maternal homocysteine concentrations, which have been associated with defective chorionic villous vascularization in women with recurrent early pregnancy loss (45). In addition, the minor allele A has been proposed to offer a protective effect against thrombosis (46). Hypercoagulation and microthrombosis at the implantation site have been hypothesized to cause implantation failure and miscarriage (40, 47). Hence, an elevated G allele frequency among women with unexplained infertility could be associated with an imbalance in coagulation at the implantation site, hampering trophoblast invasion and embryo implantation.

A major limitation of our study is the relatively low number of patients, which may have reduced the statistical power to detect associations between the studied polymorphisms and unexplained female infertility. Women diagnosed with unexplained infertility are a unique study group; however,

it is not considered to be homogeneous (48). In addition, some women with endometriosis and presenting no clinical signs of the disease could have been misdiagnosed as unexplained infertility in the absence of laparoscopic examination (49). Furthermore, some important associations between the gene variants and blood folate, vitamin B12, and homocysteine levels could have been overlooked in the situation where the majority of patients used vitamin supplements.

In conclusion, our study indicates that polymorphisms in folate-metabolizing pathway genes may contribute to fertility problems in some women with unexplained infertility. The effect could be explained by the potential of polymorphisms to alter homocysteine status, affecting the hemostatic balance, and shifting more folate cofactors to either nucleotide or methyl donor synthesis. Finally, the influence of a single variation on a phenotype may be weak, but it may become evident when coexisting with other polymorphisms or in case of folate deficiency.

Acknowledgments: The authors thank all participants, and the staff at the Department of Obstetrics and Gynecology, Karolinska University Hospital Huddinge, for collecting the material. We also thank Anna Böttiger, Örebro University, for helping with haplotype analysis.

REFERENCES

1. Tamura T, Picciano MF. Folate and human reproduction. *Am J Clin Nutr* 2006;83:993–1016.
2. Jacques PF, Bostom AG, Wilson PW, Rich S, Rosenberg IH, Selhub J. Determinants of plasma total homocysteine concentration in the Framingham Offspring cohort. *Am J Clin Nutr* 2001;73:613–21.
3. Lucock M. Folic acid: nutritional biochemistry, molecular biology, and role in disease processes. *Mol Genet Metab* 2000;71:121–38.
4. Gmyrek GB, Sozanski R, Jerzak M, Chrobak A, Wickiewicz D, Skupnik A, et al. Evaluation of monocyte chemotactic protein-1 levels in peripheral blood of infertile women with endometriosis. *Eur J Obstet Gynecol Reprod Biol* 2005;122:199–205.
5. Thaler CD, Epel D. Nitric oxide in oocyte maturation, ovulation, fertilization, cleavage and implantation: a little dab'll do ya. *Curr Pharm Des* 2003;9:399–409.
6. Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. *Reprod Biol Endocrinol* 2005;3:28.
7. Hussein MR. Apoptosis in the ovary: molecular mechanisms. *Hum Reprod Update* 2005;11:162–77.
8. Forges T, Monnier-Barbarino P, Alberto JM, Gueant-Rodriguez RM, Daval JL, Gueant JL. Impact of folate and homocysteine metabolism on human reproductive health. *Hum Reprod Update* 2007;13:225–38.
9. Mooij PN, Wouters MG, Thomas CM, Doesburg WH, Eskes TK. Disturbed reproductive performance in extreme folic acid deficient golden hamsters. *Eur J Obstet Gynecol Reprod Biol* 1992;43:71–5.
10. Boxmeer JC, Brouns RM, Lindemans J, Steegers EA, Martini E, Macklon NS, et al. Preconception folic acid treatment affects the micro-environment of the maturing oocyte in humans. *Fertil Steril* 2008;89:1766–70.
11. Chavarro JE, Rich-Edwards JW, Rosner BA, Willett WC. Use of multivitamins, intake of B vitamins, and risk of ovulatory infertility. *Fertil Steril* 2008;89:668–76.
12. Zetterberg H. Methylene tetrahydrofolate reductase and transcobalamin genetic polymorphisms in human spontaneous abortion: biological and clinical implications. *Reprod Biol Endocrinol* 2004;2:7.
13. Narayanan S, McConnell J, Little J, Sharp L, Piyathilake CJ, Powers H, et al. Associations between two common variants C677T and A1298C in the methylenetetrahydrofolate reductase gene and measures of folate metabolism and DNA stability (strand breaks, misincorporated uracil, and

- DNA methylation status) in human lymphocytes in vivo. *Cancer Epidemiol Biomarkers Prev* 2004;13:1436–43.
14. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 1995;10:111–3.
 15. Yamada K, Strahler JR, Andrews PC, Matthews RG. Regulation of human methylenetetrahydrofolate reductase by phosphorylation. *Proc Natl Acad Sci U S A* 2005;102:10454–9.
 16. Harmon DL, Woodside JV, Yarnell JW, McMaster D, Young IS, McCrum EE, et al. The common “thermolabile” variant of methylene tetrahydrofolate reductase is a major determinant of mild hyperhomocysteinemia. *QJM* 1996;89:571–7.
 17. Altmäe S, Haller K, Peters M, Hovatta O, Stavreus-Evers A, Karro H, et al. Allelic estrogen receptor 1 (ESR1) gene variants predict the outcome of ovarian stimulation in vitro fertilization. *Mol Hum Reprod* 2007;13:521–6.
 18. WHO. Laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 4th ed. Cambridge: Cambridge University Press, 1999.
 19. Böttiger AK, Hagnelius NO, Nilsson TK. Mutations in exons 2 and 3 of the FOLR1 gene in demented and non-demented elderly subjects. *Int J Mol Med* 2007;20:653–62.
 20. Böttiger AK, Hurtig-Wennlof A, Sjöström M, Yngve A, Nilsson TK. Association of total plasma homocysteine with methylenetetrahydrofolate reductase genotypes 677C>T, 1298A>C, and 1793G>A and the corresponding haplotypes in Swedish children and adolescents. *Int J Mol Med* 2007;19:659–65.
 21. Böttiger AK, Nilsson TK. Pyrosequencing assay for genotyping of the Transcobalamin II 776C>G polymorphism. *Scand J Clin Lab Invest* 2007;67:247–51.
 22. Nilsson TK, Lof-Ohlin ZM, Böttiger AK. Genotyping of the reduced folate carrier-1 c.80G>A polymorphism by pyrosequencing technology: importance of PCR and pre-PCR optimization. *Scand J Clin Lab Invest* 2008;68:166–70.
 23. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005;21:263–5.
 24. Szymanski W, Kazdepka-Zieminska A. Effect of homocysteine concentration in follicular fluid on a degree of oocyte maturity. *Ginekol Pol* 2003;74:1392–6.
 25. Ebisch IM, Peters WH, Thomas CM, Wetzels AM, Peer PG, Steegers-Theunissen RP. Homocysteine, glutathione and related thiols affect fertility parameters in the (sub)fertile couple. *Hum Reprod* 2006;21:1725–33.
 26. Pacchiarotti A, Mohamed MA, Micara G, Linari A, Tranquilli D, Espinola SB, et al. The possible role of hyperhomocysteinemia on IVF outcome. *J Assist Reprod Genet* 2007;24:459–62.
 27. Fohr IP, Prinz-Langenohl R, Bronstrup A, Bohlmann AM, Nau H, Berthold HK, et al. 5,10-Methylenetetrahydrofolate reductase genotype determines the plasma homocysteine-lowering effect of supplementation with 5-methyltetrahydrofolate or folic acid in healthy young women. *Am J Clin Nutr* 2002;75:275–82.
 28. Wang J, Huff AM, Spence JD, Hegele RA. Single nucleotide polymorphism in CTH associated with variation in plasma homocysteine concentration. *Clin Genet* 2004;65:483–6.
 29. Jongbloet PH, Verbeek AL, den Heijer M, Roeleveld N. Methylenetetrahydrofolate reductase (MTHFR) gene polymorphisms resulting in sub-optimal oocyte maturation: a discussion of folate status, neural tube defects, schizophrenia, and vasculopathy. *J Exp Clin Assist Reprod* 2008;5:5.
 30. Thaler CJ, Budiman H, Ruebsamen H, Nagel D, Lohse P. Effects of the common 677C>T mutation of the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene on ovarian responsiveness to recombinant follicle-stimulating hormone. *Am J Reprod Immunol* 2006;55:251–8.
 31. Haggarty P, McCallum H, McBain H, Andrews K, Duthie S, McNeill G, et al. Effect of B vitamins and genetics on success of in-vitro fertilisation: prospective cohort study. *Lancet* 2006;367:1513–9.
 32. Bae J, Shin SJ, Cha SH, Choi DH, Lee S, Kim NK. Prevalent genotypes of methylenetetrahydrofolate reductase (MTHFR C677T and A1298C) in spontaneously aborted embryos. *Fertil Steril* 2007;87:351–5.
 33. Reyes-Engel A, Munoz E, Gaitan MJ, Fabre E, Gallo M, Dieguez JL, et al. Implications on human fertility of the 677C→T and 1298A→C polymorphisms of the MTHFR gene: consequences of a possible genetic selection. *Mol Hum Reprod* 2002;8:952–7.
 34. Rady PL, Szucs S, Grady J, Hudnall SD, Kellner LH, Nitowsky H, et al. Genetic polymorphisms of methylenetetrahydrofolate reductase (MTHFR) and methionine synthase reductase (MTRR) in ethnic populations in Texas; a report of a novel MTHFR polymorphic site, G1793A. *Am J Med Genet* 2002;107:162–8.
 35. Chen J, Ma J, Stampfer MJ, Palomeque C, Selhub J, Hunter DJ. Linkage disequilibrium between the 677C>T and 1298A>C polymorphisms in human methylenetetrahydrofolate reductase gene and their contributions to risk of colorectal cancer. *Pharmacogenetics* 2002;12:339–42.
 36. Shi M, Caprau D, Romitti P, Christensen K, Murray JC. Genotype frequencies and linkage disequilibrium in the CEPH human diversity panel for variants in folate pathway genes MTHFR, MTHFD, MTRR, RFC1, and GCP2. *Birth Defects Res A Clin Mol Teratol* 2003;67:545–9.
 37. Ueland PM, Hustad S, Schneede J, Refsum H, Vollset SE. Biological and clinical implications of the MTHFR C677T polymorphism. *Trends Pharmacol Sci* 2001;22:195–201.
 38. Coulam CB, Jeyendran RS, Fishel LA, Roussev R. Multiple thrombophilic gene mutations are risk factors for implantation failure. *Reprod Biomed Online* 2006;12:322–7.
 39. Keijzer MB, den Heijer M, Blom HJ, Bos GM, Willems HP, Gerrits WB, et al. Interaction between hyperhomocysteinemia, mutated methylenetetrahydrofolatereductase (MTHFR) and inherited thrombophilic factors in recurrent venous thrombosis. *Thromb Haemost* 2002;88:723–8.
 40. Azem F, Many A, Ben Ami I, Yovel I, Amit A, Lessing JB, et al. Increased rates of thrombophilia in women with repeated IVF failures. *Hum Reprod* 2004;19:368–70.
 41. Rawlings ND, Barrett AJ. Evolutionary families of peptidases. *Biochem J* 1993;290:205–18.
 42. Matherly LH, Hou Z, Deng Y. Human reduced folate carrier: translation of basic biology to cancer etiology and therapy. *Cancer Metastasis Rev* 2007;26:111–28.
 43. Chango A, Emery-Fillon N, de Courcy GP, Lambert D, Pfister M, Rosenblatt DS, et al. A polymorphism (80G→A) in the reduced folate carrier gene and its associations with folate status and homocysteinemia. *Mol Genet Metab* 2000;70:310–5.
 44. Whetstone JR, Gifford AJ, Witt T, Liu XY, Flatley RM, Norris M, et al. Single nucleotide polymorphisms in the human reduced folate carrier: characterization of a high-frequency G/A variant at position 80 and transport properties of the His(27) and Arg(27) carriers. *Clin Cancer Res* 2001;7:3416–22.
 45. Nelen WL, Bulten J, Steegers EA, Blom HJ, Hanselaar AG, Eskes TK. Maternal homocysteine and chorionic vascularization in recurrent early pregnancy loss. *Hum Reprod* 2000;15:954–60.
 46. Yates Z, Lucock M. G80A reduced folate carrier SNP modulates cellular uptake of folate and affords protection against thrombosis via a non homocysteine related mechanism. *Life Sci* 2005;77:2735–42.
 47. Rey E, Kahn SR, David M, Shrier I. Thrombophilic disorders and fetal loss: a meta-analysis. *Lancet* 2003;361:901–8.
 48. Gleicher N, Barad D. Unexplained infertility: does it really exist? *Hum Reprod* 2006;21:1951–5.
 49. Olive DL, Schwartz LB. Endometriosis. *N Engl J Med* 1993;328:1759–69.