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ORIGINAL ARTICLE

Folate and vitamin B_{12} in idiopathic male infertility

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Although methylenetetrahydrofolate reductase, a folate enzyme gene, has been associated with idiopathic male infertility, few studies have examined other folate-related metabolites and genes. We investigated whether idiopathic male infertility is associated with variants in folate, vitamin B_{12} (B12) and total homocysteine (tHcy)-related genes and measured these metabolites in blood. We conducted a case–control study that included 153 men with idiopathic infertility and 184 fertile male controls recruited at the Fertility Center and Antenatal Care Center, University Hospital, Malmö and Lund, Sweden. Serum folate, red cell folate (RCF), serum B12, plasma tHcy and semen quality were measured. Subjects were genotyped for 20 common variants in 12 genes related to folate/B12/homocysteine metabolism. Metabolite concentrations and genotype distributions were compared between cases and controls using linear and logistic regression with adjustment for covariates. The phosphatidylethanolamine *N*-methyltransferase (*PEMT*) M175V and *TCblR* rs173665 polymorphisms were significantly associated with infertility ($P=0.01$ and $P=0.009$, respectively), but not with semen quality. Among non-users of supplements, infertile men had lower serum folate concentrations than fertile men (12.89 *vs.* 14.73 nmol l^{-1}; $P=0.02$), but there were no significant differences in RCF, B12 or tHcy. Folate, B12 and tHcy concentrations were not correlated with any semen parameters. This study provides little support for low folate or B12 status in the pathogenesis of idiopathic male infertility. Although additional data are needed to confirm these initial findings, our results suggest that *PEMT* and *TCblR*, genes involved in choline and B12 metabolism, merit further investigation in idiopathic male infertility.
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INTRODUCTION

Idiopathic male infertility is an important contributor to infertility overall.[1] Abnormal folate metabolism has been proposed as a factor in male infertility. This is biologically plausible because folate-derived one-carbon units are critical in DNA synthesis and the regulation of DNA transcription *via* methylation, two key processes in spermatogenesis.[2]

The 677C>T variant in the gene for methylenetetrahydrofolate reductase (*MTHFR*), an essential enzyme for providing methyl groups for the regulation of DNA transcription,[3] has been studied extensively as a risk factor with mixed results.[4–11] A recent meta-analysis concluded that *MTHFR* 677C>T was a risk factor for idiopathic male infertility.[12] However, only one investigation by Lee *et al.*[8] presented actual serum folate concentrations for study subjects, an important factor in determining what the physiological effect of the *MTHFR* variant would be. Almost no studies have examined blood vitamin B_{12} (B12) concentrations, although B12 is an essential component of one-carbon metabolism, being a cofactor in the folate-dependent conversion of homocysteine to methionine; and few have examined plasma total homocysteine (tHcy) concentrations despite the fact that elevated tHcy is a marker for poor folate and B12 function.

We investigated the association between folate/B12 metabolism and idiopathic male infertility by measuring circulating concentrations of serum folate, red cell folate (RCF), B12 and plasma tHcy, and by genotyping variants of 12 folate/B12/homocysteine metabolism-related genes in infertile and fertile men.

MATERIALS AND METHODS

Study population

Between March 2003 and August 2008, 200 infertile men were recruited from the Reproductive Medicine Center at Skåne University Hospital in Malmö. The men had to be between 20 and 45 years of age with partners less than 40 years of age. They were required to have had regular sexual intercourse without contraception for $\geqslant 1$ year without achieving a pregnancy, to answer questions about their medical and behavioral history, and to provide blood and semen samples. They were examined for other possible causes of infertility by a research nurse and were surveyed about their partner's reproductive health history to rule out female infertility. The work-up of the female partner followed standard clinical criteria. In all women, gynecological history, hormonal evaluation and ovarian ultrasound were performed. The fallopian tubes were assessed by X-ray or ultrasound in cases where damage

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to the fallopian tubes was suspected (previous pregnancy, abdominal/genital surgery or infection/inflammation).

Excluded from the analysis were men not having regular, unprotected intercourse (n=5), who withdrew consent (n=3), who did not provide adequate screening information or biological specimens (n=22), who had partners with disturbances in ovulation (n=3), and who had histories of cancer (n=1), cryptorchidism (n=4), Klinefelter's syndrome (n=2) or microdeletions (n=6). One man was dropped when he impregnated his partner. Other exclusion criteria were a history of vasectomy, obstructive azoospermia, hypogonadotropic hypogonadism or mumps orchitis, but we did not observe any men with these conditions. After exclusions 153 infertile men remained.

We recruited 200 fertile men from among couples attending prenatal care clinics in Lund and Malmö. Fertile study participants were required to have achieved at least one pregnancy with a female partner, stopped practicing birth control to achieve the present pregnancy, to be within the same age ranges as infertile couples and to have achieved the present pregnancy in <12 months of unprotected intercourse. Fertile men were excluded because they did not fulfill the age criteria (n=1), had partners who did not fulfill the age criteria (n=2), did not discontinue contraception to achieve their current pregnancy (n=3), did not provide adequate screening information or biological specimens (n=4), had histories of cryptorchidism (n=5) or were part of a couple that had prior infertility treatment (n=1). Other exclusion criteria were a history of cancer and treatment of mumps orchitis, but we did not observe any men with these conditions. After exclusions 184 fertile men remained.

Study design

Cases (infertile men) and fertile controls responded to a questionnaire for ascertainment of reproductive, medical and recent dietary history and provided blood and semen for analysis. The time between semen sample collection and blood drawing ranged from 0 to 3 months. Men were asked if they took any vitamins. Those who took any supplements containing folic acid were considered vitamin users for this analysis. This study was approved by the Institutional Review Boards of Lund University, Sweden and the National Institutes of Health, the United States. Written informed consent was obtained from all subjects.

Semen analysis

Investigators followed the World Health Organization's guidelines for collecting and analyzing semen samples.[13] Skåne University Hospital is a reference laboratory for the European Society of Human Reproduction and Embryology and the Nordic Association for Andrology Quality Control Programme. Semen volume, sperm concentration, total number of sperm per ejaculate, and sperm motility and morphology were determined. The strict criteria for morphology were applied.[13] Sperm DNA fragmentation was evaluated using the sperm chromatin structure assay.[14]

Blood measurements

Serum and plasma were stored at −20 °C. Frozen samples were randomized and investigators performing the measurements were blinded to the case–control status of the samples. Microbiological assays were used to measure concentrations of folate and B12 in serum and folate in erythrocytes.[15,16] Plasma tHcy was measured by automated immunoassay using fluorescent polarization.[17]

Genotyping

Genomic DNA extracted from blood samples was genotyped for 20 single nucleotide polymorphisms (SNPs) in 12 genes related to folate, B12 and homocysteine metabolism using a competitive allele-specific PCR genotyping system (KBiosciences, Herts, UK). SNPs were selected because they were reported to have an effect on folate, B12 or tHcy levels or were identified as important in the medical literature. Quality control procedures included repeat genotyping of all SNPs for 17 subjects and a repeat DNA extraction for 20 subjects. Genotype concordance was 100% for repeat genotyping and 99.8% for genotypes from repeat DNA extractions. The proportion of genotypes that were successfully called was >95.8% for all 20 SNPs. For each SNP, a test of Hardy–Weinberg equilibrium was performed separately for case and control subjects. Two SNPs were not in Hardy–Weinberg equilibrium among controls: *MTHFR* 1298A>C (rs1801131), P=0.0071; and *MTRR* S175L (rs1532268), P=0.0076. There was no deviation from Hardy–Weinberg equilibrium among cases.

Statistical analysis

The characteristics of cases and controls were compared using unpaired two sample t-tests, Pearson's chi-square test and Fisher's exact test. P values <0.05 were considered statistically significant. Linear regression models were used to compare semen quality between users and non-users of vitamins, and mean folate, B12 and tHcy concentrations between cases and controls, while adjusting for covariates. Linear regression was also used to examine associations between semen quality and folate, B12 and tHcy concentrations. Logistic regression was used to calculate adjusted odds ratios (OR) and 95% confidence intervals (CI) for the association between risk of infertility (case–control status) and metabolite concentrations among non-users of vitamins, and for case–control comparisons of SNP genotype distributions. For SNPs that showed an association with infertility status, linear regression was used to compare semen quality among the genotype groups separately for cases and controls, and after restricting the study sample to non-users of vitamins. Age (continuous), education (elementary, high school, beyond high school), smoking (yes/no), length of abstinence, parents born in Sweden (yes/no) and use of vitamins (use of folic acid containing vitamins, use of other vitamins, no use) were included as covariates in linear and logistic regression models. SAS 9.1.3 (SAS Institute, Cary, NC, USA) was used for all statistical analyses.

RESULTS

Demographic characteristics of the 184 fertile controls and 153 infertile cases by use of folic acid-containing vitamin status are presented in **Table 1**. Infertile men and their partners were older and the men had a higher mean body mass index. The fertile controls were significantly more educated. Duration of abstinence before semen sample collection was longer in the infertile group. The proportions that used vitamins containing folic acid and that smoked were similar in the two groups.

As anticipated, infertile cases had poorer overall semen quality than fertile controls. Infertile subjects had lower mean semen concentration, sperm number, percentage total progressive sperm and percentage normal sperm morphology (all P<0.05). Infertile subjects had higher percentages of non-motile sperm, higher mean semen volume and more incidences of DNA fragmentation than fertile controls (all P<0.05) (data not shown).

When we compared the infertile subjects who used and did not use folic acid-containing vitamins, there were no significant differences in semen quality after adjustment (**Table 2**). The linear regression results

Table 1 Baseline characteristics of cases and controls

Characteristics	*Non-users of vitamins*			*Users of vitamins with folic acid*			*All cases* (n=153)	*All controls* (n=184)	P *value*[a]
	Cases (n=110)	*Controls* (n=123)	P *value*[a]	*Cases* (n=32)	*Controls* (n=34)	P *value*[a]			
Age (year)[b]	38.51±4.98	35.30±4.46	<**0.0001**	37.91±3.64	35.94±4.97	0.074	33.35±4.48	32.2±4.46	**0.036**
Partner's age (year)[b]	30.57±4.12	30.01±4.00	0.29	31.97±3.50	30.09±2.90	**0.020**	31.00±4.00	30.07±3.86	**0.031**
Body mass index (kg m^{-2})[b]	25.98±3.80	24.95±3.30	**0.030**	26.56±4.33	24.71±3.43	0.060	26.07±3.84	24.93±3.28	**0.0045**
Education[c]									
Elementary	12 (10.91)	3 (2.44)	**0.0004**	5 (15.63)	0 (0.00)	**0.019**	18 (11.76)	6 (3.26)	<**0.001**
High school	55 (50.00)	40 (32.52)		12 (37.50)	9 (26.47)		70 (45.75)	56 (30.43)	
Beyond high school	42 (38.18)	78 (63.41)		15 (46.88)	25 (73.53)		64 (41.83)	120 (65.22)	
Missing[d]	1 (0.91)	2 (1.63)		0 (0.00)	0 (0.00)		1 (0.65)	2 (1.09)	
Parents born in Sweden[c]	70 (63.64)	93 (75.61)	0.056	24 (75.00)	18 (52.94)	0.077	101 (68.24)	130 (71.04)	0.077
Currently using cigarettes[c]	23 (20.91)	22 (17.89)	0.83	5 (15.63)	3 (8.82)	0.47	29 (19.21)	25 (13.74)	0.47
Currently using snuff[c]	22 (20.00)	33 (26.83)	0.47	3 (9.38)	6 (17.65)	0.38	27 (18.12)	41 (22.91)	0.38
Abstinence time (day)[c]									
0–1	5 (4.55)	14 (11.38)	<**0.0001**	0 (0.00)	6 (17.65)	<**0.0001**	5 (3.27)	27 (14.67)	<**0.0001**
2–3	28 (25.45)	35 (28.46)		10 (31.25)	6 (17.65)		38 (24.83)	49 (26.63)	
4–5	47 (42.73)	10 (8.13)		11 (34.38)	3 (8.82)		64 (41.83)	15 (8.15)	
>5	17 (15.45)	8 (6.50)		9 (28.13)	2 (5.88)		30 (19.61)	11 (5.98)	
Missing[d]	13 (11.82)	56 (45.53)		2 (6.25)	17 (50.00)		16 (10.46)	82 (44.57)	
Vitamin use[c]									
Vitamins with folic acid	—	—	—	—	—	—	32 (20.92)	34 (18.48)	0.12
Vitamins without folic acid	—	—		—	—		11 (7.19)	26 (14.13)	
No vitamin use	—	—		—	—		110 (71.90)	123 (66.85)	
Missing[d]	—	—		—	—		0 (0.00)	1 (0.54)	

[a] Comparison between cases and controls using unpaired two sample *t*-tests for age, partner's age and body mass index, using Pearsons's Chi-square test for education, abstinence time and vitamin use, and using Fisher's exact test for proportion with parents born in Sweden and use of cigarettes and snuff.
[b] Values are mean±s.d.
[c] Values are *n* (%).
[d] Observations with missing values were not included when performing statistical tests.

Table 2 Semen quality in cases and controls by vitamin use

	Sperm concentration (10^6 *sperm* ml^{-1})	*Sperm number (in millions)*	*Semen volume (ml)*	*Sperm DNA fragmentation (%)*	*Non-motile sperm (%)*	*Total progressive sperm (%)*	*Sperm with normal morphology (%)*
Cases							
Non-users of vitamins (*n*=110)[a]	27.98±36.63	101.36±140.53	4.21±2.07	21.84±10.89	47.35±19.40	34.01±19.11	2.71±3.04
Users of vitamins with folic acid (*n*=32)[a]	34.22±37.31	129.46±145.38	4.77±1.90	21.79±13.07	41.23±16.21	37.38±18.06	3.23±3.52
P value (adjusted for age, smoking, abstinence time)[b]	0.18	0.069	0.16	0.49	0.15	0.42	0.47
P value (adjusted for age, smoking, abstinence time, education)[b]	0.19	0.081	0.24	0.48	0.19	0.50	0.47
Controls							
Non-users of vitamins (*n*=123)[a]	81.93±69.39	333.05±109.94	3.59±1.47	12.30±7.51	24.85±10.21	59.67±12.31	7.65±3.68
Users of vitamins with folic acid (*n*=34)[a]	73.69±41.77	316.56±91.35	3.44±1.82	9.88±7.01	23.26±11.44	61.15±12.85	8.03±3.51
P value (adjusted for age, smoking, abstinence time)[b]	0.79	0.28	0.84	0.39	0.57	0.95	0.81
P value (adjusted for age, smoking, abstinence time, education)[b]	0.65	0.18	0.73	0.51	0.63	0.93	0.78

[a] Values are mean±s.d.
[b] Comparison between vitamin users and non-users separately for cases and controls using linear regression models with adjustment for covariates.

did not show significant associations between semen parameters and folate, B12 and tHcy concentrations in cases or controls.

In both the case and control groups, subjects who reported taking vitamin supplements had higher folate and B12 concentrations and lower tHcy concentrations than non-users of supplements. All the differences between users and non-users were statistically significant ($P<0.05$) (data not shown).

The following analyses focused on non-users of vitamins on the assumption that, if low folate concentrations were an important factor, the use of vitamins would very likely obscure the effect. Among non-users of vitamins, serum folate and RCF concentrations were significantly lower in the infertile than the fertile group ($P=0.0063$ and $P=0.044$, respectively) after adjusting for age, smoking and abstinence time (**Table 3**). When education was added to the regression model, serum folate remained significant ($P=0.022$); RCF did not. As expected with lower folate concentrations, the tHcy concentration was higher in infertile than fertile men, although the difference was not statistically significant (**Table 3**). B12 concentrations were lower in the infertile than the fertile group, but the difference was not statistically significant. In the logistic regression analysis, serum folate was a significant predictor of being a case (OR: 0.40; 95% CI: 0.18–0.90); RCF, B12 and tHcy were not.

Table 4 lists the 12 genes associated with folate/B12/tHcy metabolism. Male infertility was positively associated with being homozygous for the choline pathway enzyme gene phosphatidylethanolamine *N*-methyltransferase (*PEMT*) M175V minor V allele (OR: 7.91; 95% CI: 1.60–39.05; $P=0.01$) and negatively associated with the B12 transcobalamin receptor gene *TCblR* (also known as *CD320*) variant rs173665 in the heterozygous subjects (OR: 0.34; 95% CI: 0.15–0.77; $P=0.009$). No cases homozygous for the minor A allele were found. Homozygosity for the *MTHFR* 1298A>C minor C allele was associated with infertility status; however, this SNP was not in Hardy–Weinberg equilibrium among controls. No statistically significant genotype results remained after correction for multiple comparisons using the Bonferroni method. There was no association between semen quality measures or folate/B12/tHcy levels and *PEMT* M175V or *TCblR* rs173665 genotypes.

MTHFR 677C>T was not associated with male infertility in our study population. To determine whether this was due to higher folate concentrations in our population compared to previous studies, we repeated the analysis in those whose serum folate concentration was below the study population median (14.70 nmol l^{-1}). The results were unchanged.

DISCUSSION

Among non-users of vitamins, the infertile men in our study had significantly lower serum folate concentrations than fertile controls. RCF concentrations were not significantly lower in cases after adjusting for education, suggesting that lower socioeconomic status-related dietary factors could explain the difference. Cases and controls did not differ in B12 and tHcy concentrations. No metabolite concentrations were correlated significantly with any semen parameters. Of the folate/B12/homocysteine-related genes that we studied, variants for two: the transcobalamin receptor gene, *TCblR*, and the choline pathway gene, *PEMT*, were related to infertility before correction for multiple comparisons.

Only a few folate-, B12- or homocysteine-related polymorphisms have been investigated as risk factors for idiopathic male infertility. We attempted to confirm many of them. A recent meta-analysis of *MTHFR* 677C>T reported a significant association.[12] We found no association even when we limited our analysis to subjects with folate concentrations below the median; however, the upper bound of our 95% CI is within the range of the reported effect. A few studies have looked for an association between male infertility and variants in two key enzyme genes involved in methylation and homocysteine metabolism, *MTR* and *MTRR*, with mixed results.[8,11] We did not find SNPs in either *MTR* or *MTRR* to be associated with infertility; we also confirmed

Table 3 Folate, vitamin B_{12} and homocysteine concentrations in cases and controls

	Serum folate (nmol l^{-1})	*Serum vitamin B_{12} (pmol l^{-1})*	*Plasma total homocysteine (μmol l^{-1})*	*Red cell folate (nmol l^{-1})*
Comparison of metabolite concentrations between cases and controls[a]				
Non-users of vitamins				
Cases ($n=110$)[c]	12.89±5.92	281.18±84.18	13.25±5.86	649.14±203.61
Controls ($n=123$)[c]	14.73±6.00	300.23±91.76	12.77±7.59	714.48±223.36
P value (adjusted for age, smoking, abstinence time)	**0.0063**	0.44	0.077	**0.044**
P value (adjusted for age, smoking, abstinence time, education)	**0.022**	0.80	0.10	0.16
Users of vitamins with folic acid				
Cases ($n=32$)[c]	26.81±18.59	355.96±124.35	10.57±2.77	984.32±333.63
Controls ($n=34$)[c]	25.46±14.94	346.44±104.98	10.29±1.90	1022.15±315.90
P value (adjusted for age, smoking, abstinence time)	0.84	0.48	0.73	0.55
P value (adjusted for age, smoking, abstinence time, education)	0.51	0.46	0.83	0.47
Risk of infertility (case–control analysis) associated with metabolite concentration, among non-users of vitamins[b]				
OR (95% CI): adjusted for age, smoking, abstinence time	**0.36 (0.16, 0.78)**	0.66 (0.23, 1.85)	3.18 (0.81, 12.47)	0.28 (0.08, 1.02)
P value	**0.0094**	0.43	0.097	0.054
OR (95% CI): adjusted for age, smoking, abstinence time, education	**0.40 (0.18, 0.90)**	0.87 (0.29, 2.64)	3.01 (0.78, 11.62)	0.32 (0.08, 1.30)
P value	**0.027**	0.81	0.11	0.11

[a] Comparison using linear regression models; model covariates are listed as adjustment factors.
[b] Logistic regression used to generate odds ratios (OR) and 95% confidence intervals (CI); each metabolite was examined in a separate model; model covariates are listed as adjustment factors.
[c] Values are presented as mean ± s.d..

Table 4 Comparison of genotype distributions between control and infertile men

Gene symbol; name	*Protein function*	*SNP (description)*	*Alleles*[a]	*Case genotypes*[b]	*Control genotypes*[b]	*Adjusted OR for infertility (95% CI)*[c]	
						Heterozygous	*Homozygous for minor allele*[d]
BHMT; betaine-homocysteine methyltransferase	Converts betaine and homocysteine to dimethylglycine and methionine	rs3733890 (R239Q)	G/A	84/54/11	106/65/13	0.81 (0.41, 1.61)	0.63 (0.19, 2.10)
TCblR (also known as *CD320*); transcobalamin receptor	Facilitates the uptake of transcobalamin-bound vitamin B_{12} into tissues	rs2227288 (G>C: 5′ near gene)	G/C	120/26/4	144/36/3	1.34 (0.55, 3.25)	0.81 (0.09, 7.55)
		rs173665 (C>T: 3′ near gene)	G/A	125/24/0	141/35/3	**0.34 (0.15, 0.77)**	—
FOLH1 (also known as *GCPII*); folate hydrolase (folylpolyglutamate carboxypeptidase)	Hydrolyzes folate polyglutamates	rs61886492 (H475Y)	C/T	130/17/0	168/12/0	1.79 (0.62, 5.18)	—
MTHFD1; methylenetetrahydrofolate dehydrogenase ($NADP^+$-dependent) 1	C1-synthase trifunctional enzyme catalyzing the interconversion of 1-carbon derivatives of tetrahydrofolate	rs2236225 (R653Q)	C/T	53/71/27	64/83/36	1.24 (0.63, 2.44)	1.22 (0.50, 2.95)
MTHFR; methylenetetrahydrofolate reductase (NAD(P)H)	Converts 5,10-methylenetetra-hydrofolate to 5-methyltetrahydrofolate	rs1801133 (677C>T)	C/T	73/63/13	94/73/15	0.93 (0.49, 1.75)	1.44 (0.45, 4.57)
		rs1801131 (1298A>C)	A/C	58/77/11	87/62/27	1.52 (0.79, 2.94)	**0.24 (0.06, 0.95)**
MTR; methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase)	Converts homocysteine and 5-methyltetrahydrofolate to methionine and tetrahydrofolate	rs1805087 (D919G)	A/G	100/41/6	116/57/8	1.12 (0.58, 2.19)	1.52 (0.27, 8.41)
MTRR; methionine synthase reductase	Regenerates functional methionine synthase *via* reductive methylation	rs1801394 (M22I)	G/A	50/68/32	60/88/32	0.73 (0.37, 1.47)	1.43 (0.59, 3.44)
		rs1532268 (S175 L)	G/A	59/70/19	76/69/36	0.83 (0.42, 1.65)	0.44 (0.18, 1.08)
PEMT; phosphatidylethanolamine N-methyltransferase	Converts phosphatidyl-ethanolamine to phosphatidylcholine by sequential methylation	rs897453 (V58I)	G/A	40/74/34	52/85/44	0.81 (0.38, 1.72)	0.57 (0.24, 1.36)
		rs7946 (M175V)	T/C	77/58/14	94/79/8	0.87 (0.46, 1.64)	**7.91 (1.60, 39.05)**
		rs12325817 (744C>G)	C/G	42/65/39	48/87/43	0.79 (0.37, 1.68)	0.93 (0.40, 2.18)
SHMT1; serine hydroxymethyl-transferase 1 (soluble)	Converts serine and tetrahydrofolate to glycine and 5,10-methylene tetrahydrofolate	rs1979277 (L474F)	G/A	60/64/27	86/81/15	0.99 (0.51, 1.92)	1.93 (0.76, 4.88)
RFC1 (also known as *SLC19A1*); reduced folate carrier	Facilitates the transport of reduced folates into cells	rs1051266 (R27H)	G/A	48/76/23	57/95/27	0.79 (0.39, 1.59)	0.86 (0.33, 2.26)
PCFT (also known as SLC46A1);proton-coupled folate transporter	Transport of folate across cell membranes under specific PH conditions	rs9909629 (A>T: 5′ near gene)	A/T	134/12/0	153/26/0	0.35 (0.12, 1.03)	—
		rs11080058 (G>A: 5′ near gene)	G/A	73/67/8	94/72/17	1.10 (0.58, 2.09)	0.39 (0.12, 1.32)
TCN2; transcobalamin II	Binds cobalamin and mediates its transport into cells	rs9606756 (I23V)	A/G	113/29/4	142/34/4	0.92 (0.41, 2.09)	2.86 (0.30, 26.85)
		rs1801198 (P259R)	C/G	45/71/28	57/92/29	0.76 (0.37, 1.56)	0.64 (0.25, 1.67)
		rs9621049 (S348F)	C/T	112/33/3	139/35/4	1.23 (0.56, 2.69)	1.84 (0.15, 22.29)

[a] Major allele is listed first.
[b] Genotype values are numbers of individuals with homozygous major allele/heterozygous/homozygous minor allele.
[c] Homozygous for major allele was reference category; logistic regression analysis adjusted for age, education, smoking, use of vitamins, abstinence time, and parents born in Sweden.
[d] Homozygote odds ratios were not obtained when there were no cases and/or controls that were homozygous for the minor allele.

that *RFC1* (also known as *SLC19A1*) R27H was not associated with infertility.[18]

The other eight genes we investigated in the areas of folate, B12 and homocysteine metabolism have not, to our knowledge, been investigated previously. Variants in these enzyme genes are potentially important because they affect homocysteine and choline metabolism (*BHMT*, *PEMT*), folate transport and action (*FOLH1*, *MTHFD1*, *SHMT1*, *PCFT*) and B12 uptake (*TCblR*) and transport (*TCN2*).

Of the 20 SNPs that we studied, *PEMT* M175V and *TCblR* rs173665 showed associations with infertility. The *PEMT* variant that was significantly more common in cases is noteworthy because it is a loss of function mutation that has been shown to cause increased tHcy when folate status is marginal.[19] This result suggests that choline metabolism may play a role in idiopathic male infertility. The *TCblR* variant is also of interest because the minor A allele was also found to be protective against neural tube defects in a recent population-based study.[20] None of the variants that we found to be associated with infertility was associated with significantly poorer semen quality suggesting that these findings require additional investigation.

Previous studies have produced mixed results regarding the role of folate in male infertility. A randomized controlled trial performed by Landau *et al.*[21] reported that folic acid supplementation does not improve overall semen quality. Another randomized controlled trial of folic acid and zinc found a 74% increase in the sperm count in the men who took folic acid supplements.[22] An uncontrolled study of folinic acid, 15 mg daily for 3 months, showed a significant improvement in spermatozoa number and motility.[23]

Somewhat surprisingly, blood folate, B12 and tHcy concentrations in infertile men have not been studied often and the results have been conflicting. One study reported that serum folate and B12 were lower and tHcy was higher in infertile subjects, but a *P* value was reported only for tHcy.[10] In contrast, a trial to determine whether folic acid could improve sperm parameters reported that fertile and subfertile men had almost identical folate concentrations (18.0 nmol l^{-1}) prior to treatment.[24] A third study reported no difference in blood folate, B12 and tHcy values between their fertile and infertile groups.[25] Our data do not show an association between B12 or tHcy and semen parameters. After adjusting for education, only serum folate, not RCF, was significantly lower in infertile men not taking folic acid supplements. The inclusion of RCF is a major advantage over most previous studies because it provides a long-term (90–120 days) measure of folate status.

Several limitations of our study should be noted. The infertile men were undergoing fertility evaluations and provided semen samples on a different day than the blood samples. Fertile men provided semen and blood samples on the same day. The lifetime of an erythrocyte is 90–120 days and spermatogenesis takes 72 days, so our RCF measure would reflect the period of spermatogenesis. Abstinence time was not available for 45% of controls. Strengths of our study include the extensive genotyping and biochemical data on a relatively large sample of men carefully screened for known causes of infertility.

In conclusion, this study provides little support for the importance of low folate or B12 in the pathogenesis of idiopathic male infertility. The role of genetic variants (*PEMT* and *TCblR*) in choline and B12 metabolism merits further investigation.

AUTHOR CONTRIBUTIONS

RJL, AG, HS, DW-S and JLM conceived and designed the study. AG, HS, DW-S and AMM collected the data and performed laboratory analyses. LEM, CQ and TCC performed statistical analyses. LEM and JLM wrote the manuscript with input from all co-authors. All authors revised the manuscript for intellectual content and approved the final version.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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