

Can the genetic polymorphisms of the folate metabolism have an influence in the polycystic ovary syndrome?

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ABSTRACT

Objective: To investigate the association of the genetic variants of the folate metabolism genes (*MTHFR* C677T; *MTHFR* A1298C; *MTR* A2756G; *MTRR* A66G and *RFC-1* A80G) with the development of polycystic ovary syndrome (PCOS). **Subjects and methods:** This study included 203 women (99 women with PCOS and 104 controls). The genotyping was performed by PCR-RFLP. Chi-squared test and multiple logistic regression were used in the statistical analysis. Haplotype analysis was conducted using the SNPstat program. The results were presented in *odds ratio* (OR) and confidence interval of 95% (CI-95%), with a significance level of 5% ($p \leq 0.05$). **Results:** The genotypic distribution of the *RFC-1* A80G polymorphism showed significant difference between the two groups, showing that the heterozygous genotype (AG genotype) was most frequent in controls. The polymorphic homozygous (GG genotype) of *MTRR* A66G polymorphism were most frequent in controls. The T-C haplotype *MTHFR* C677T and A1298C polymorphisms were more frequent in the control group (OR = 0.19; CI 95% — 0.04 to 0.93 e $p = 0.042$). The multivariate analysis evidenced that family history of PCOS was more frequent in the PCOS group (OR = 3.29; CI 95% — 1.48 to 7.31; $p = 0.003$). **Conclusion:** In our casuistry, the polymorphic homozygous of *MTRR* A66G polymorphism gene and heterozygous of *RFC-1* A80G polymorphism gene, the haplotype T-C C677T and A1298C polymorphisms of *MTHFR* gene, can be associated with protective factors for the disease.

Keywords

Polycystic ovary syndrome; folic acid; polymorphism

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INTRODUCTION

The Polycystic Ovary Syndrome (PCOS) is characterized by the presence of polycystic ovaries morphology, oligo or chronic anovulation and hyperandrogenism (1,2).

The etiology of PCOS is unclear, but this syndrome is a multifactorial disease that results from complex interactions between genetic and environmental factors (3). One study performed in 1968 showed the

possible effect of folic acid on ovarian function in rats, evidencing that a deficiency or an excess of folic acid partially inhibited ovulation (4). The effect of folate deficiency was also studied on the cytomorphology and the kinetics of proliferation of ovarian granulosa cells, and the epithelial cells lining the uterus, cervix and vagina of six sexually mature female rhesus monkey and showed degeneration of Graffian follicles with an increase in atretic and cystic follicles, accompanied by

a depletion of granulosa cells and reduction or even absence of corpora lutea (5). The homocysteine is produced in all cells and can be produced by folate metabolism that is reduced to tetrahydrofolate which is then converted to 5, 10-methylenetetrahydrofolate (6). Several studies confirmed the presence of increased serum Hcy concentration in PCOS patients (7).

The enzymes methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR), methionine synthase reductase (MTRR) and reduced folate carrier 1 (RFC-1) participate in the folate metabolism. Single nucleotide polymorphisms (SNPs) can lead to modifications in enzymatic activities involved in the metabolic pathway of folate and may be implicated in the etiology of the PCOS, mainly in epigenetic changes such as alterations in DNA methylation pattern (4-6,8). Several literature reports showed that the *MTHFR* C667T and A1298C, *MTR* A2756G, *MTRR* A66G and *RFC-1* A80G SNPs are genetic factors that can modify the activities of the folate metabolism enzymes (9-12).

Several studies evaluated the association between PCOS and *MTHFR* polymorphisms and found contradictory results. However, the study of Fu and colleagues showed that the T allele of *MTHFR* C677T polymorphism is associated with the PCOS development (13).

Considering the evidences showed in several studies indicating that the genetic polymorphisms can have influence in PCOS and the lack of studies in the Brazilian population, the aim of this study was to investigate the association of the genetic variants of the folate metabolism genes (*MTHFR* C677T – rs1801133, *MTHFR* A1298C – rs1801131, *MTR* A2756G – rs1805087, *MTRR* A66G – rs1801394 and *RFC-1* A80G – rs1051266 polymorphisms with the development of PCOS).

SUBJECTS AND METHODS

Casuistry

This case-control study was approved by the Ethics Committee of the Federal University of Triângulo Mineiro (UFTM) (Protocol 1796), with all patients signing the Informed Consent Term.

All the women in this study were availed by specialists of a research group from the Gynecology and Obstetrics and the Adrenal and Gonadal Endocrinology sectors of the Clinics Hospital of UFTM, Uberaba, Minas Gerais,

Brazil. Participants inclusion in the study occurred in the period from 2012 to 2016.

This study included 203 women (99 women with PCOS and 104 controls). PCOS patients were diagnosed using the Rotterdam criteria. The control group consisted of women in reproductive age, with no current or superior use of contraceptives for 3 months and no history or signs of PCOS. The exclusion criteria consisted of: presence of Cushing's syndrome, congenital adrenal hyperplasia, thyroid dysfunction, hyperprolactinemia, diabetes mellitus, adrenal or ovarian tumors, use of anti-androgens, statins, glucocorticoids or medications for infertility.

Molecular analysis

The genomic DNA was extracted from peripheral blood leukocytes according to the method of Phenol: Chloroform: Isoamyl (14) and storage at 4°C. The genotyping of the *MTHFR* C677T (rs1801133), *MTHFR* A1298C (rs1801131), *MTR* A2756G (rs1805087), *MTRR* A66G (rs1801394) and *RFC-1* A80G (rs1051266) polymorphisms were analyzed by Polymerase Chain Reaction and Restriction Fragment Length Polymorphism (PCR-RFLP) using protocols previously described (15-18).

Statistical analysis

Statistical analyses were performed using Stacts Direct and BioEstat version 3.0. Chi-squared test and multiple logistic regression were used in the statistical analysis.

Comparisons between the differences of genotypic distributions and Hardy-Weinberg equilibrium (HWE) were made by chi-square test. The multiple regression logistic test was used to determine the effects of variables in PCOS. The model included smoking habit (reference: no), alcohol habit (reference: no), family history of PCOS (reference: no), and the polymorphisms (reference: homozygous wild type).

The binary logistic regression model adjusted for age was used to assess the association between polymorphisms and development of PCOS using the SNPStats program (available at: http://bioinfo.iconcologia.net/SNPstats_web). The effects of the polymorphisms were evaluated in the following models: (1) codominant (homozygous wild type *vs* heterozygous *vs* polymorphic homozygote); (2) dominant (homozygous wild type *vs* heterozygous *plus* polymorphic homozygous); (3) recessive (polymorphic homozygous *vs* homozygous

wild type *plus* heterozygous) and (4) overdominant (homozygous wild type *plus* polymorphic homozygous *vs* heterozygous). The *MTHFR* haplotype was inferred using the SNPStats program, which checked population frequency estimates of the haplotype.

The software G POWER 3.1 was used to verify the statistical power of the study and presented 95%, post hoc.

The results were shown as odds ratios (OR) and 95% confidence interval (CI 95%). The level of significance was set at 5% ($p \leq 0.05$).

RESULTS

In this study, sociodemographic data, including age, smoking and alcohol habits and historical family PCOS, were collected. Also, information as absence of pregnancy and menstrual irregularities, comorbidities, use of hormonal or non-hormonal medication, hyperandrogenic aspects, weight, height, BMI were collected and are shown in the Table 1. The clinical data were collected by follow-up in the medical records.

In the univariate analysis, the genotype distribution of *RFC-1* A80G polymorphism showed significant difference between the two groups ($\chi^2 = 8.42$; $p = 0.01$). For the other polymorphisms, the genotype distribution has not shown statistic difference between

the cases and controls for *MTHFR* C677T ($\chi^2 = 1.14$; $p = 0.56$), *MTHFR* A1298C ($\chi^2 = 0.04$; $p = 0.98$), *MTR* A2756G ($\chi^2 = 0.13$; $p = 0.57$) and *MTRR* A66G polymorphisms ($\chi^2 = 3.47$; $p = 0.18$).

With regard to the HWE, the genotypic frequencies of the *MTHFR* C677T ($\chi^2 = 0.11$; $p = 0.74$ cases; $\chi^2 = 0.03$; $p = 0.86$ controls) and *MTR* A2756G polymorphisms ($\chi^2 = 0.0006$; $p = 0.98$ cases; $\chi^2 = 0.33$; $p = 0.57$ controls) were found in equilibrium. The *MTRR* A66G and *RFC-1* A80G polymorphisms showed equilibrium in controls ($\chi^2 = 3.28$; $p = 0.07$; $\chi^2 = 0.05$; $p = 0.83$, respectively), but disequilibrium in cases ($\chi^2 = 5.55$; $p = 0.02$; $\chi^2 = 14.01$; $p = 0.0002$, respectively). The *MTHFR* A1298C polymorphism showed disequilibrium in cases and controls ($\chi^2 = 8.33$; $p = 0.004$; $\chi^2 = 8.73$; $p = 0.003$, respectively).

Table 2 shows the haplotypes that were constructed with the analysis of the *MTHFR* polymorphisms genes studied (C677T and A1298C). The C-A, T-A and C-C haplotype frequencies were similar between both groups. However, the T-C haplotype frequency showed significant difference between the PCOS group and control group (OR = 0.19; CI 95%: 0.04-0.93 and $p = 0.042$), with higher frequency in the control group.

In our study, the analysis of the association of the five genetic polymorphisms (the *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, *MTRR* A66G and *RFC-1* A80G) is presented in Table 3. The genotypes of 99 women with PCOS and 90 controls were adjusted for age according to the heritable models. The SNP *MTRR* A66G in the recessive model (OR = 0.34; CI 95%: 0.12-0.96; $p = 0.035$) showed a higher frequency of the recessive genotype in the control group. The SNP *RFC-1* A80G in the codominant model (OR = 0.42; CI 95%: 0.20-0.87; $p = 0.049$) and overdominant model (OR = 0.46; CI 95%: 0.25-0.88; $p = 0.017$) had higher frequency of the heterozygous genotype in the control group. These results may suggest an association of these polymorphisms as protective factors in PCOS development.

Table 1. Characterization of the study group with women with PCOS and controls

Variables	PCOS	Controls
	N/n (% or mean)	N/n (% or mean)
Age (years)	97 (26.06 ± 7.32*)	99 (31.73 ± 9.65*)
Absence of pregnancy	96/76 (79.17)	99/38 (38.38)
Menstrual irregularities	97/34 (35.05)	98/06 (06.12)
Family history of PCOS	95/37 (38.95)	100/20 (20.00)
Smoking habit	95/07 (07.37)	100/25 (25.00)
Alcohol habit	97/25 (25.77)	100/29 (29.00)
Comorbidities	94/44 (46.81)	98/29 (29.59)
Use of hormonal medication	95/54 (56.84)	100/09 (09.00)
Hirsutism	93/52 (55.91)	100/05 (05.00)
Acne	93/61 (65.59)	100/29 (29.00)
Skin Oiliness	93/73 (78.49)	100/49 (49.00)
Loss of hair	92/58 (63.04)	99/35 (35.35)
Weight (meters)	89 (1.63 ± 0.07*)	96 (1.62 ± 0.06*)
Height (kg)	91 (79.70 ± 22.45*)	96 (67.34 ± 14.65*)
BMI (kg/m ²)	89 (29.72 ± 8.82*)	96 (25.44 ± 4.86*)
Overweight or obesity	/58 (65.17)	/48 (50.00)

* Mean of variable and standard deviation; N: total of women with the analyzed variable; n: number of women with the variable analyzed.

Table 2. Haplotype frequencies of the *MTHFR* C677T and A1298C polymorphisms gene in women with PCOS and controls

Haplotype	PCOS Frequency	Control Frequency	O.R (CI 95%)	p value
C-A	0.38	0.39	1.00	-
T-A	0.29	0.29	1.07 (0.62 to 1.82)	0.82
C-C	0.31	0.26	1.53 (0.91 to 2.56)	0.11
T-C	0.02	0.07	0.19 (0.04 to 0.93)	0.042*

OR: odds ratio; CI: confidence interval. * $p < 0.05$.

Table 3. Association of the *MTHFR* C677T, *MTHFR*A1298C, *MTR*A2756G, *MTRR*A66G and *RFC-1* A80G polymorphisms gene with PCOS, adjusted for age

Model	Genotype	Case N (%)	Control N (%)	OR (CI 95%)	p value
<i>MTHFR</i> C677T					
Codominant	C/C	44 (47.30)	39 (43.30)	1.00	0.32
	C/T	42 (45.20)	39 (43.30)	0.90 (0.47-1.73)	
	T/T	07 (07.50)	12 (13.40)	0.45 (0.15-1.30)	
Dominant	C/C	44 (47.30)	39 (43.30)	1.00	0.45
	C/T-T/T	49 (52.70)	51 (56.70)	0.79 (0.43-1.46)	
Recessive	C/C-C/T	86 (92.50)	78 (86.70)	1.00	0.14
	TT	07 (07.50)	12 (13.40)	0.47 (0.17-1.30)	
Overdominant	C/C-T/T	51 (54.80)	51 (56.70)	1.00	0.87
	C/T	42 (45.20)	39 (43.30)	1.05 (0.57-1.95)	
<i>MTHFR</i> A1298C					
Codominant	A/A	48 (51.60)	48 (53.30)	1.00	0.60
	A/C	29 (31.20)	27 (30.00)	1.43 (0.70-2.94)	
	C/C	16 (17.20)	15 (16.70)	1.26 (0.54-2.95)	
Dominant	A/A	48 (51.60)	48 (53.30)	1.00	0.33
	A/C-C/C	45 (48.40)	42 (46.70)	1.36 (0.73-2.55)	
Recessive	A/A-A/C	77 (82.80)	75 (83.30)	1.00	0.81
	C/C	16 (17.20)	15 (16.70)	1.10 (0.49-2.48)	
Overdominant	A/A-C/C	64 (68.80)	63 (70.00)	1.00	0.39
	A/C	29 (31.20)	27 (30.00)	1.35 (0.68-2.67)	
<i>MTR</i> A2756G					
Codominant	A/A	59 (63.40)	54 (60.00)	1.00	0.71
	A/G	31 (33.30)	31 (34.40)	0.86 (0.45-1.64)	
	G/G	03 (03.20)	05 (05.60)	0.56 (0.12-2.60)	
Dominant	A/A	59 (63.40)	54 (60.00)	1.00	0.53
	A/G-G/G	34 (36.60)	36 (40.00)	0.82 (0.44-1.53)	
Recessive	A/A-A/G	90 (96.80)	85 (94.40)	1.00	0.49
	GG	03 (03.20)	05 (05.60)	0.59 (0.13-2.70)	
Overdominant	A/A-G/G	62 (66.70)	59 (65.60)	1.00	0.73
	A/G	31 (33.30)	31 (34.40)	0.89 (0.47-1.70)	
<i>MTRR</i> A66G					
Codominant	A/A	33 (35.50)	25 (27.80)	1.00	0.09
	A/G	54 (58.10)	51 (56.70)	0.81 (0.41-1.60)	
	G/G	06 (06.50)	14 (15.60)	0.29 (0.09-0.92)	
Dominant	A/A	33 (35.50)	25 (27.80)	1.00	0.28
	A/G-G/G	60 (64.50)	65 (72.20)	0.69 (0.36-1.34)	
Recessive	A/A-A/G	87 (93.50)	76 (84.40)	1.00	0.035*
	GG	06 (06.50)	14 (15.60)	0.34 (0.12-0.96)	
Overdominant	A/A-G/G	39 (41.90)	39 (43.30)	1.00	0.77
	A/G	54 (58.10)	51 (56.70)	1.10 (0.59-2.04)	
<i>RFC-1</i> A80G					
Codominant	A/A	36 (38.70)	25 (27.80)	1.00	0.049*
	A/G	28 (30.10)	45 (50.00)	0.42 (0.20-0.87)	
	G/G	29 (31.20)	20 (22.20)	0.79 (0.35-1.78)	
Dominant	A/A	36 (38.70)	25 (27.80)	1.00	0.066
	A/G-G/G	57 (61.30)	65 (72.20)	0.54 (0.28-1.05)	
Recessive	A/A-A/G	64 (68.80)	70 (77.80)	1.00	0.49
	GG	29 (31.20)	20 (22.20)	1.28 (0.64-2.56)	
Overdominant	A/A-G/G	65 (69.90)	45 (50.00)	1.00	0.017*
	A/G	28 (30.10)	45 (50.00)	0.46 (0.25-0.88)	

Table 4 shows the results of multiple logistic regression analysis in 85 women with PCOS and 84 controls to determine the effects of the variables. The smoking habit was more frequent in the control group (OR = 0.13; CI 95%: 0.04-0.41; p = 0.004). The PCOS family history was more frequent in the case group (OR = 3.29; CI 95%: 1.48-7.31; p = 0.003).

DISCUSSION

This study evaluated the association of five genetic polymorphisms involved in the folate metabolism (*MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, *MTRR* A66G and *RFC-1* A80G) in women with PCOS and controls in a Brazilian population. The analysis of polymorphisms of the folate metabolic pathway enzymes genes can provide relevant data on how these

genetic variants may alter the structural and functional properties of the enzymes, and result in important alterations. The enzymes methylenetetrahydrofolate reductase enzyme (*MTHFR*), methionine synthase (*MTR*) and methionine synthase reductase (*MTRR*), are involved in folate metabolism, and polymorphisms identified in their genes can lead to the functional alterations and may alter the folate metabolism (7,19).

The present study tested HWE for the genotype frequencies of the five polymorphisms analyzed. The polymorphisms *MTHFR* A1298C were in disequilibrium in the cases and controls, and the polymorphisms *MTRR* A66G and *RFC-1* A80G presented disequilibrium in the case group. When the HWE was tested, the disequilibrium between cases and controls may be due to the random selection of the individuals studied, the adopted disease model

Table 4. Relation between risk factors for PCOS development and the genetic polymorphisms of the *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, *MTRR* A66G and *RFC-1* A80G in women with PCOS and controls

Variable analyzed	PCOS N (%)	Control N (%)	O.R (CI 95%)	p value
Risk factors				
Smoking Habit				
Yes	05 (05.88)	23 (27.38)	0.13 (0.04-0.41)	0.004*
No	80 (94.11)	61 (72.62)		
Alcohol Habit				
Yes	20 (23.53)	24 (28.57)	0.96 (0.44-2.07)	0.91
No	65 (76.47)	60 (71.43)		
Family History of PCOS				
Yes	31 (36.47)	14 (16.67)	3.29 (1.48-7.31)	0.003*
No	54 (63.53)	70 (83.33)		
GENETIC POLYMORPHISMS				
<i>MTHFR</i> A1298C				
AA	42 (49.41)	46 (54.76)	1.06 (0.53-2.13)	0.87
AC / CC	43 (50.59)	38 (45.24)		
<i>MTHFR</i> C677T				
CC	40 (47.06)	37 (44.05)	1.01 (0.50-2.01)	0.98
CT / TT	45 (52.94)	47 (55.95)		
<i>MTR</i> A2756G				
AA	52 (61.18)	51 (60.71)	0.85 (0.43-1.68)	0.64
AG / GG	33 (38.82)	33 (39.29)		
<i>MTRR</i> A66G				
AA	32 (37.65)	23 (27.38)	0.67 (0.33-1.36)	0.26
AG / GG	53 (62.35)	61 (72.62)		
<i>RFC-1</i> A80G				
AA	35 (41.18)	24 (28.57)	0.52 (0.25- 1.06)	0.07
AG / GG	50 (58.82)	60 (71.43)		

OR: odds ratio; CI: confidence interval. * p ≤ 0.05.

and/or random changes in the genotype frequencies due to sampling errors (genetic drift). When there is no balance among the cases, it may be related to the disease (with a statistical difference in the genotypic frequency) or deletion of the deleterious allele by the action of natural selection (with no statistical difference in genotypic frequency). When the control individuals are in HW disequilibrium, it may mean that there is a protective effect (20-22).

In our results, there were no association between PCOS and the SNPs C677T and A1298C *MTHFR* gene. Table 5 summarizes the case-control studies with PCOS and *MTHFR* gene polymorphisms in different populations and shows that the results of the association of these polymorphisms are contradictory.

This study evaluated the relationship between haplotypes of the *MTHFR* gene polymorphisms and PCOS. The haplotype T-C was more frequent in the controls. Other studies have not made this inference.

The present study has not found any association between the SNP *MTR* A2756G and PCOS. This result is in agreement with one study available in the literature that analyzed 46 women with PCOS and 25 controls, in two ethnicities: Caucasian (25 PCOS and 16 controls) and South Asian (21 PCOS and 9 controls), and the relation of *MTR* A2756G polymorphism with the biochemical, hematological and endocrinological parameters and plasma levels of homocysteine (8).

No association between SNP *MTRR* A66G and PCOS were observed in our study. However, the recessive inheritance model showed that GG genotype is more frequent in the control group and this result can suggest that the recessive homozygous can be related as a protective factor in our studied population. Another

study showed association between the lower level of the total cholesterol and LDL cholesterol when compared to AA and AG genotypes (30).

In our study, the univariate analysis of the SNP *RFC-1A80G* showed significant difference between the cases and controls. The codominant and overdominant inheritance models showed that the AG genotype was more frequent in controls. Results of our study suggest that heterozygous individuals can show protector effect against PCOS. There are few studies in the literature about the *RFC-1A80G* polymorphism gene and some comorbidities, like hyperhomocysteinemia (31).

Regarding casuistry in this study, the multivariate analysis evidenced that family history of PCOS was more frequent in women with PCOS. This result shows that genetic factors can be related to the etiology of PCOS, which is in accordance with other studies. A study carried out in 2006 evidenced that the syndrome has a hereditary basis, and that genetic factors are related with PCOS development (32). Other research, in 2010, investigated the genes susceptible to the development of PCOS in 502 families with daughters and sisters with PCOS and it concluded that some genes are related to the risk of developing the syndrome (33). Therefore, this information constitutes an important basis for justification of studies of susceptible genes in PCOS. This analysis also showed that smoking habits was more frequent in controls; this finding can be due to the treatment of PCOS in the case of female smokers includes quitting smoking. Discontinuation of cigarette smoking may result in decreased levels of androgens in the circulation. Likewise, smoking is a contraindication to the use of oral contraceptives, which are often prescribed to patients with PCOS (34,35).

Table 5. Case-control studies with PCOS and *MTHFR* gene polymorphisms

Authors	Country	SNPs	Cases	Controls	Conclusion
Wu and cols., 2016 (23)	China	<i>MTHFR</i> C677T <i>MTHFR</i> A1298C	244	257	No association
Szafarowska and cols., 2016 (24)	Poland	<i>MTHFR</i> C677T <i>MTHFR</i> A1298C	76	56	No association
Carlus and cols., 2016 (25)	India	<i>MTHFR</i> C677T	261	256	No association
Qi and cols., 2015 (26)	China	<i>MTHFR</i> C677T <i>MTHFR</i> A1298C	115	58	Association with <i>MTHFR</i> C677T and PCOS No association with <i>MTHFR</i> A1298C
Idali and cols., 2012 (27)	Iran	<i>MTHFR</i> C677T <i>MTHFR</i> A1298C	71	106	No association with <i>MTHFR</i> C677T and A1298C and PCOS Association with two SNPs <i>MTHFR</i> in women with PCOS plus recurrent pregnancy loss
Karadeniz and cols., 2010 (28)	Turkey	<i>MTHFR</i> C677T	86	70	No association
Choi and cols., 2009 (29)	Korea	<i>MTHFR</i> C677T	227	115	No association

In this study, we did not collect the level of homocysteine and data reference of the food supplementation from female participants. It was not possible to conduct the analysis of the biochemical factors and the SNPs studied because the laboratory data was performed using medical records. These facts constituted limitations to our study. However, this study is very relevant because there are few studies regarding polymorphisms in PCOS in the Brazilian population and this is the first study that analyzed the polymorphisms of folate metabolism in the syndrome.

In conclusion, in relation to casuistry, the polymorphic homozygous of MTRR A66G polymorphism gene and heterozygous of RFC-1 A80G polymorphism gene, the haplotype T-C C677T and A1298C polymorphisms of MTHFR gene, can be associated with protective factors for the disease.

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