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Homocysteine Levels and C677T Polymorphism of Methylenetetrahydrofolate Reductase in Women with Polycystic Ovary Syndrome

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The aim of this study was to investigate the homocysteine (Hcy) levels and the C677T polymorphism of 5,10-methylenetetrahydrofolate reductase (MTHFR), a crucial factor of the Hcy metabolism in young women with polycystic ovary syndrome (PCOS).

Seventy young women with PCOS and another 70 healthy women with low folate intake were enrolled. Cases and controls were matched for age, body mass index, and allele frequency. Hcy, vitamin B_{12}, and folate levels were measured, and a genetic analysis of 5,10-MTHFR at nucleotide 677 was performed in all subjects.

No difference in mean Hcy levels was observed between PCOS women in comparison to the control group. Considering the different MTHFR polymorphism, no significant difference was found in serum Hcy levels between subjects with PCOS and controls showing CC (10.4 ± 3.1 *vs.* 9.7 ± 2.9 μmol/liter ± SD) and CT genotypes (10.9 ± 3.8 *vs.* 11.0 ± 3.2 μmol/liter ± SD). In subjects with a TT homozygous state, a significant ($P < 0.05$) difference was observed between PCOS and control women (11.5 ± 3.9 *vs.* 22.0 ± 7.8 μmol/liter ± SD).

In conclusion, our data show that in PCOS women, the serum Hcy levels are normal, and the C677T polymorphism of MTHFR does not influence the Hcy levels like in controls. (*J Clin Endocrinol Metab* 88: 673–679, 2003)

POLYCYSTIC OVARY SYNDROME (PCOS) is one of the most common endocrine diseases of women of fertile age, affecting up to 10% of women in reproductive age, and is characterized by hyperandrogenism, chronic anovulatory cycles, and oligomenorrhea or amenorrhea (1). At present, PCOS is considered not only a disease that influences fertility but also a plurimetabolic syndrome (2).

Recent data have shown an increased prevalence of cardiovascular disease (CVD) in PCOS women (3–6), and several studies have evaluated whether there is a higher cardiovascular morbidity and mortality in these patients (7, 8).

Elevated plasma homocysteine (Hcy) levels are considered to be an independent risk factor for CVD (9–11). A strong association between plasma Hcy concentrations and CVD has been observed (12–16). The mechanism of the Hcy action on the cardiovascular system, furthermore, is still unknown.

Circulating Hcy levels could be influenced by many determinants (12). Genetic factors play an important role in the metabolic pathway of Hcy synthesis. Enzymatic defects caused by genetic mutations induce a significant increase in Hcy concentrations (17). In the general population, the most common cause of abnormal serum Hcy levels seems to be a reduced efficiency of methylenetetrahydrofolate reductase (MTHFR), an enzyme involved in the folate-dependent remethylation of Hcy to methionine (18). The C677T mutation, *i.e.* alanine to valine substitution at nucleotide 677, in MTHFR gene causes an impairment of its enzymatic activity (19–26). When C677T mutation is present in a homozygous state, this polymorphism results in a variant of MTHFR enzyme, referred to as the thermolabile MTHFR (18), and in elevated circulating total Hcy levels (15–16, 27).

At present, no data are available in literature regarding the relationship between serum Hcy levels and MTHFR polymorphism in women with PCOS.

In this view, the aim of this study was to investigate the serum Hcy levels and the C677T polymorphism of MTHFR in young women with PCOS.

Patients and Methods

The procedures used were in accordance with the guidelines of the Helsinki Declaration on human experimentation. The study was approved by the Institutional Review Board of the University of Naples. The purpose of the protocol was explained to both the patients and control women, and written consent was obtained from them before beginning the study.

Patients

Seventy young women [age, 22.5 ± 4.3 (SD) yr; range, 18–27 yr] with PCOS were enrolled for the study. PCOS was defined according to clinical (Ferriman-Gallwey score > 8; and/or oligomenorrhea or amenorrhea), biological (LH/FSH ratio > 2; hyperandrogenism), and ultrasonographic findings (28, 29).

Another 70 healthy young volunteer females, matched for age, body mass index (BMI), and allele frequency, were enrolled and considered

Abbreviations: A, Androstenedione; AUC, area under curve; BMI, body mass index; CBS, cystathione-β-synthase; CI, confidence interval; CV, coefficient(s) of variation; CVD, cardiovascular disease; DHEAS, dehydroepiandrosterone sulfate; E_2, 17β-estradiol; Hcy, homocysteine; MTHFR, methylenetetrahydrofolate reductase; OGTT, oral glucose tolerance test; 17-OHP, 17-hydroxyprogesterone; P, progesterone; PCOS, polycystic ovary syndrome; PRL, prolactin; T, testosterone.

as the control group. Their healthy state was determined by medical history, physical and pelvic examination, and complete blood chemistry. Their normal ovulatory state was confirmed by transvaginal ultrasonography and plasma progesterone (P) assay detected during the luteal phase of the cycle.

To avoid dietary interference, only women with a low folate intake were enrolled. In the present study, we considered women at low folate intake when the serum folate levels were below 12.5 nmol/liter (30, 31).

Exclusion criteria for all subjects included pregnancy, hypothyroidism, current use of vitamins, current or previous (within the last 6 months) use of oral contraceptives, glucocorticoids, antiandrogens, ovulation induction agents, antidiabetic and antiobesity drugs, or other hormonal drugs. None of the patients were affected by any neoplastic, metabolic, and cardiovascular disorder or other concurrent medical illness (*i.e.* diabetes, renal disease, and malabsorptive disorders). All subjects were nonsmokers and had a normal physical activity, and none drank alcoholic beverages.

The cases and the controls were genetically unrelated.

Study protocol

At study entry, all subjects underwent venous blood drawing for complete hormonal assays, lipid profile, glucose, insulin, and Hcy levels, and MTHFR genetic study.

Glucose and insulin values were detected also after oral glucose tolerance test (OGTT).

All blood samples were obtained in the morning between 0800 h and 0900 h after a 3-d, 300-g carbohydrate diet, an overnight fasting, and resting in bed during early follicular phase (d 2–5) of the spontaneous or P-induced menstrual cycle.

During the same visit, all subjects underwent transvaginal ultrasonography, anthropometric measurements, including BMI and waist to hip ratio, systolic and diastolic blood pressure, echocardiographic assessment, and echocolor-Doppler with evaluation of intima media thickness.

In the present study, only the data regarding the serum Hcy levels and the genetic evaluation of MTHFR will be shown and discussed.

Biochemical assay

A butterfly needle was inserted into an antecubital vein, and an iv saline infusion was given at a rate of approximately 50 ml/h. The subjects were kept supine throughout the infusion period and were not allowed to smoke, sleep, or drink alcoholic or caffeinated beverages.

Thirty minutes after the needle insertion, basal blood samples were obtained to evaluate complete hormonal assays. On these samples the following hormonal serum levels were measured: LH, FSH, 17β-estradiol (E_2), P, total testosterone (T), androstenedione (A), dehydroepiandrosterone sulfate (DHEAS), prolactin (PRL), TSH, and SHBG. All blood samples for each woman were assayed in duplicate determinations and immediately centrifuged, and the serum was stored at −80 C until analysis. The mean of two hormonal results was calculated.

Plasma PRL, LH, TSH, FSH, E_2, P, T, A, and DHEAS were measured by specific RIA, as previously described (32). Serum 17-hydroxyprogesterone (17-OHP) levels were determined using a RIA (DSL 5000, Diagnostic Systems Laboratories, Inc., Webster, TX) with a sensitivity of 0.5 nmol/liter and intra-assay and interassay coefficients of variation (CV) of 8.9 and 9.0%, respectively. SHBG levels were measured using an immunoradiometric assay (Radim S.p.A, Pomezia, Rome, Italy) with a sensitivity of 2.5 nmol/liter and intra-assay and interassay CV of 5.1% and 5.2%, respectively.

Glucose and insulin concentrations were measured also 30 min after insertion of the iv catheter to detect the fasting levels (time 0) before OGTT. Successively, each subject received 75-g glucose load orally. Further blood samples (10 ml each) were obtained at 30-min intervals for the following 3 h during the infusion period (times 30, 60, 90, 120, 150, and 180 min), and glucose and insulin concentrations were determined.

Plasma glucose levels were determined by the glucose oxidase method on a Beckman Glucose Analyzer (Beckman Coulter, Inc., Fullerton, CA), with a sensitivity of 0.3 mmol/liter and intra-assay and interassay CV of 1.0% and 1.2%, respectively. Serum insulin was measured by a solid-phase chemiluminescent enzyme immunoassay using commercially available kits (Immunolite, Diagnostic Products, Los Angeles, CA) with a sensitivity of 2.0 μU/ml and intra-assay and interassay CV of 5.5% and 5.8%, respectively.

Glucose tolerance was assessed by World Health Organization criteria (33).

In the PCOS and control groups, the glucose and insulin response to OGTT was analyzed by calculating the area under curve (AUC). The AUCs for glucose ($AUC_{glucose}$) and insulin ($AUC_{insulin}$) were determined according to the mathematical method described by Tai (34) for the metabolic curves. The $AUC_{glucose}/AUC_{insulin}$ ratio was also calculated in each subject (35).

Homocysteine and vitamins assays

Serum Hcy levels were measured by HPLC using a C-18 reverse phase 25 × 0.46-cm column and fluorescence detection (Beckman Gold System, Beckman Coulter, Inc.), and an isocratic buffer consisting of 0.1 M acetate buffer (pH 4.0; containing 20 ml methanol/liter buffer). Detection was accomplished on a Shimadzu RF535 fluorometric detector (Shimadzu Corp., Kyoto, Japan; emission λ, 385 nm; excitation λ, 515 nm). Ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate was used as a derivative agent of the Hcy thiol residue. Hcy sensitivity of the assay was greater than 0.25 μmol/liter of serum. Quantitation accuracy was granted by the use of an internal standard (acetyl-cysteine), and the inter- and intra-assay CV were 2% and 1%, respectively.

Vitamin B_{12} and folate were analyzed by capillary electrophoresis (P/ACE 5000 system, Beckman Coulter, Inc., Palo Alto, CA). Separations were accomplished by the use of a 75-μm (internal diameter) silica-fused capillary with a length to detector of 50 cm. The capillary was filled with a 10 mM Tris-sodium phosphate buffer (pH 7.56). The applied voltage was 20 kV, and detection was 200 nm. The inter- and intra-assay CV for vitamin B_{12} assay were 7.4% and 6.7%, respectively, and for folate assay, 4.1% and 4.2%, respectively.

DNA analysis

The DNA analysis was performed in the MeriGen Molecular Biology Laboratory (Naples, Italy). Blood samples were collected in tubes containing disodium-EDTA as anticoagulant and stored at 4 C until DNA extraction. DNA was extracted by the salt phenol chloroform method from the buff coat cells (36). The extracted DNA was stored at −20 C until analysis.

Briefly the primers used (5′TGAAGGAGAAGGTGTCTGCGGGA 3′ exonic and 5′AGGACGGTGCGGTGAGAGTG 3′ intronic) for the analysis of the A → V change generated, by PCR, a 198-bp DNA fragment. The substitution created a *Hin*fI recognition sequence that digested the 198-bp fragment into 175 and 23-bp fragments. MTHFR genotype was determined by *Hin*fI digestion of the PCR products, separated by 2% agarose gel electrophoresis, and visualized under UV light after ethidium staining, as described by Frosst *et al.* (18).

Genotypes were expressed as CC for homozygous normal, CT for heterozygous, and TT for homozygous mutant.

Statistical analysis

Statistical analysis was performed using the SPSS 9.0 (SPSS, Inc., Chicago, IL) package. Continuous data were expressed as mean ± SD. A P value less than 0.05 was considered statistically significant.

The characteristics of the patients and the mean plasma hormone concentrations between the two clinical groups were compared by Student's t test for unpaired data and between and within the different groups of MTHFR genotypes with the ANOVA.

Student's t test for unpaired data was also used to evaluate the differences in mean Hcy, vitamin B_{12}, folate, glucose, and insulin levels in PCOS and control groups.

A linear regression analysis was performed to study the relationship between serum Hcy levels and vitamin B_{12} and folate concentrations.

Allelic and genotypic frequencies were determined from observed genotype counts, and the expectations of the Hardy-Weinberg equilibrium were evaluated by χ^2 analysis. Differences in the genotype distribution between different groups were assessed by Pearson's χ^2 test of heterogeneity.

ANOVA was used to compare the difference in mean Hcy, vitamin

B_{12}, folate, $AUC_{glucose}$, $AUC_{insulin}$, and $AUC_{glucose}/AUC_{insulin}$ ratio after OGTT between and within the different groups of MTHFR genotypes.

The correlation between $AUC_{glucose}$, $AUC_{insulin}$, and $AUC_{glucose}/AUC_{insulin}$ ratio and Hcy levels in PCOS and healthy women, according to different MTHFR genotype, was studied using a linear regression analysis.

Results

The demographic and hormonal data of the PCOS and control groups are reported in Table 1. In the PCOS group, LH, 17-OHP, T, A, E_2, DHEAS, and SHBG were significantly ($P < 0.05$) different in comparison with healthy women. The fasting insulin levels were significantly ($P < 0.05$) higher in PCOS than in control women, whereas no difference in fasting glucose concentrations was observed between groups. No significant differences were detected in serum vitamin B_{12} and folate or in Hcy levels between PCOS and healthy women.

A significant ($P < 0.05$) inverse correlation was observed between serum Hcy levels and vitamin B_{12} ($r = -0.42$ and $r = -0.45$ for PCOS and control groups, respectively) and folate concentrations ($r = -0.39$ and $r = -0.41$ for PCOS and control groups, respectively).

The mutation rate of C677T in MTHFR is shown in Table 2. The allelic distribution of MTHFR genotypes was in Hardy-Weinberg equilibrium for both groups of women. There was a similar genotype distribution between the PCOS and control groups (Table 2).

No difference in demographic data was detected between or within the different groups of MTHFR genotypes (data not shown). In comparison to the control group, the PCOS women (also according to the MTHFR genotypes) maintained the same differences in hormonal data observed in Table 1 (data not shown). In particular, no differences in serum vitamin B_{12} and folate levels were observed in PCOS and control women according to the genotype groups (Table 3).

In Fig. 1, the serum Hcy levels are shown in PCOS and control women according to the different groups of MTHFR polymorphism.

In the control group, the mean serum Hcy levels were significantly ($P < 0.05$) higher in women with the TT genotype compared with CC and/or CT genotypes (22.0 ± 7.8 *vs.* 9.7 ± 2.9 and 11.0 ± 3.2 μmol/liter, respectively; Fig. 1). On the contrary, no significant difference was detected between the three groups of different polymorphisms in PCOS patients (10.4 ± 3.1, 10.9 ± 3.8, and 11.5 ± 3.9 μmol/liter, for CC, CT, and TT genotypes, respectively; Fig. 1).

No significant difference was found in serum Hcy levels between subjects with PCOS and controls for CC genotype [10.4 ± 3.1 (SD) *vs.* 9.7 ± 2.9 μmol/liter] and for CT heterozygosity (10.9 ± 3.8 *vs.* 11.0 ± 3.2 μmol/liter; Fig. 1). A significant ($P < 0.05$) difference was observed between subjects with PCOS and controls with TT homozygosity (11.5 ± 3.9 *vs.* 22.0 ± 7.8 μmol/liter; Fig. 1).

After OGTT, four (5.7%) women with PCOS were diagnosed to have glucose intolerance, whereas only one (1.4%) woman in the control group had glucose intolerance. $AUC_{insulin}$ and $AUC_{glucose}/AUC_{insulin}$ ratio were significantly ($P < 0.05$) higher in PCOS than in control women, whereas no statistical difference was observed in $AUC_{glucose}$ (Table 1).

No difference was observed between and within MTHFR genotypes in fasting glucose levels and in $AUC_{glucose}$ after OGTT (Table 4). The fasting insulin levels were significantly ($P < 0.05$) higher in PCOS women in comparison with controls without a difference within and between MTHFR genotypes (Table 4).

The $AUC_{insulin}$ and $AUC_{glucose}/AUC_{insulin}$ ratio were also significantly ($P < 0.05$) higher in PCOS women than in control women in all MTHFR genotypes without difference between MTHFR genotypes (Table 4).

A significant ($P < 0.05$) relation was detected between Hcy levels and fasting insulin, $AUC_{insulin}$, and $AUC_{glucose}/$

TABLE 1. Clinical and biochemical data of women with PCOS and controls

	PCOS (n = 70)	Controls (n = 70)
Age (yr)	22.5 ± 4.3	21.9 ± 3.2
BMI (kg/m^2)	24.1 ± 3.1	23.8 ± 3.7
Waist/hip ratio	0.88 ± 0.1	0.85 ± 0.3
Ferriman-Gallwey score	12.1 ± 1.1[a]	5.2 ± 0.3
FSH (IU/liter)	9.5 ± 1.2	9.8 ± 1.3
LH (IU/liter)	30.6 ± 3.7[a]	14.6 ± 1.5
PRL (ng/ml)	10.8 ± 0.7	11 ± 0.8
E_2 (pmol/liter)	167.9 ± 18.1[a]	149.8 ± 17.3
P (nmol/liter)	1.1 ± 0.5[a]	1.7 ± 0.7
17-OHP (nmol/liter)	43.9 ± 0.2[a]	42.7 ± 0.3
T (nmol/liter)	2.8 ± 0.3[a]	1.7 ± 0.2
A (nmol/liter)	7.14 ± 0.7[a]	4.44 ± 0.9
DHEAS (μmol/liter)	4416 ± 401[a]	3618 ± 343
SHBG (nmol/liter)	29.1 ± 5.2[a]	50.3 ± 8.4
IGF-I (nmol/liter)	47.6 ± 2.1[a]	33.5 ± 8.5
Vitamin B_{12} (ng/ml)	377.4 ± 101.5	384.5 ± 128.7
Folate (nmol/liter)	8.6 ± 2.4	8.5 ± 2.3
Hcy (μmol/liter)	11.3 ± 3.7	12.2 ± 4.5
Fasting glucose (mmol/liter)	6.9 ± 3.1	5.6 ± 2.9
Fasting insulin (μU/ml)	21.1 ± 6.7[a]	7.8 ± 2.1
OGTT		
$AUC_{glucose}$	1254 ± 562	1216 ± 458
$AUC_{insulin}$	6063 ± 1474[a]	2587 ± 397
$AUC_{glucose}/AUC_{insulin}$ ratio	0.19 ± 0.33[a]	0.49 ± 0.40

Data are expressed as mean ± SD.
[a] $P < 0.05$ *vs.* control group.

TABLE 2. Frequency of different genotypes in PCOS and control women

	Genotype		
	CC	CT	TT
PCOS (n = 70)	16 (22.9)	41 (58.5)	13 (18.6)
Controls (n = 70)	17 (24.3)	38 (54.3)	15 (21.4)

Data are expressed as number (%).

TABLE 3. Serum vitamin B_{12} and folate levels in PCOS and control women according to MTHFR genotype

Genotype	PCOS		Control	
	Vitamin B_{12} (ng/ml)	Folate (nmol/liter)	Vitamin B_{12} (ng/ml)	Folate (nmol/liter)
CC	350.1 ± 98.2	8.9 ± 2.3	377.2 ± 128.3	8.6 ± 2.3
CT	396.3 ± 102.3	8.4 ± 2.2	369.7 ± 114.2	8.3 ± 2.1
TT	378.9 ± 99.6	8.7 ± 2.4	400.6 ± 97.2	8.9 ± 2.2

Data are expressed as mean ± SD.

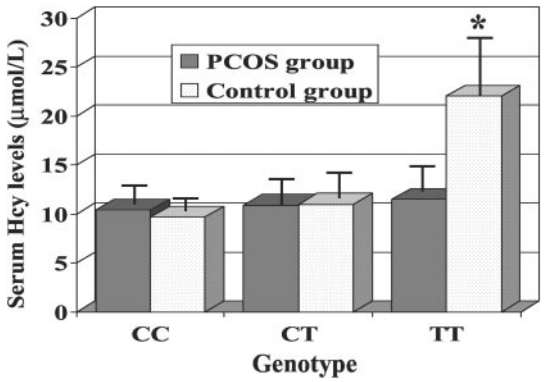

FIG. 1. Serum Hcy levels according to the genotype distribution in PCOS and controls. Values are expressed as mean ± SD. *, $P < 0.05$ *vs.* all genotypes in PCOS women and *vs.* CC and CT genotypes in control women.

$AUC_{insulin}$ ratio in PCOS women (r = 0.41, r = 0.52, and r = 0.58 for fasting insulin, $AUC_{insulin}$, and $AUC_{glucose}/AUC_{insulin}$ ratio, respectively) and healthy women (r = 0.42, r = 0.49, and r = 0.56, for fasting insulin, $AUC_{insulin}$, and $AUC_{glucose}/AUC_{insulin}$ ratio, respectively). In PCOS and control women, these significant relations were maintained in CC and CT, but not in TT genotypes (Table 5).

Discussion

Only within the past 5 yr has Hcy taken its place among other major risk factors such as cholesterol, smoking, and obesity; it is now widely accepted as a major independent risk factor for cardiovascular, cerebrovascular, and peripheral vascular disease (21).

Several *in vitro* (37–45) and *in vivo* (46–50) studies have shown that Hcy acts on the cardiovascular system with a direct toxicity on the endothelium. In particular, Hcy increases DNA synthesis in vascular smooth muscle cells inducing its proliferation (48, 40–45), whereas it blocks the regeneration of endothelial cells (47) and causes oxidation of low lipoprotein (49–51). One of the most frequent causes of increased plasma levels of Hcy is a decreased activity of 5,10-MTHFR (18, 23, 26). In the original reports by Kang *et al.* (23, 52), it had been suggested that there is a variant of the MTHFR enzyme characterized by a thermolability and a specific enzyme activity of approximately 50% of the normal activity. This variant varies significantly in populations from different geographic areas (23, 53, 54) and has been observed in about 17% of subjects with CVD (55) and 28% of patients with hyperhomocysteinemia and premature vascular disease (56).

In our study, we observed a high prevalence of homozygosity for the C677T mutation as detected by other studies in the Italian population (57–59). Furthermore, it is still not clear whether homozygosity for MTHFR C677T mutation is a real risk factor for CVD. This contention has been supported by some studies (23, 52, 55–57, 60–64), but rejected by others (24, 65–72).

Currently, it is unknown whether Hcy may play a role in the increased cardiovascular risk demonstrated in PCOS women. Moreover, no study has focalized the attention on the relationship between MTHFR polymorphism and Hcy levels in women with PCOS.

Our data show that the Hcy levels in PCOS women are in the normal range and similar to those obtained in our control group. These findings are in contrast to the current literature (73, 74). In fact, in these last studies (73, 74), elevated serum Hcy concentrations were found in PCOS women, suggesting that an alteration in Hcy metabolism may play a role in the increased cardiovascular risk associated with PCOS. Furthermore, there are many methodological differences. First of all, in our study the assessment of Hcy concentrations was performed using a more sensitive and specific assay, that is HPLC. In addition, our sample of women was larger and younger in comparison to the subjects studied by other authors (73, 74). Finally, our study population was based on a sample of subjects in Hardy-Weinberg equilibrium for the allelic distribution of MTHFR genotypes, whereas nongenetic evaluation was performed by Yarali *et al.* (73) and Loverro *et al.* (74).

Our findings are similar to those obtained by Sills *et al.* (27). In this last study (27), serum androgens and fasting insulin levels, but not Hcy concentrations, were significantly higher in women with polycystic ovaries. Furthermore, also in this last study (27), no DNA analysis was performed.

Our study confirms that the homozygosity of C677T MTHFR mutation is associated with high levels of Hcy in healthy women (23, 75). Furthermore, a lack of significant association between the MTHFR mutation and high levels of Hcy in PCOS women was observed, not precluding the possibility that the C677T mutation could be a marker for vascular disease in PCOS women because this was not the aim of our study.

No association was found between genotype and fasting Hcy in subjects with plasma folate levels at or above the median (15 nmol/liter; Ref. 76). Plasma Hcy concentrations are not different between genotypes when folate concentrations are above the median, whereas elevated Hcy concentrations have been observed in subjects with TT genotype when folate levels are below the median or the lowest quartile (68).

Many studies have shown that plasma Hcy concentration is inversely related to blood levels of vitamin B_{12} and folate (77). The highest Hcy concentrations observed in TT women are due to a low dietary intake of vitamins and to a major susceptibility to this inadequate alimentary support (31, 78). On the contrary, when folate consumption is high, the TT genotype has little effect on Hcy levels (18).

On the basis of these considerations, to obtain a homogeneous group of subjects and to eliminate the confounding factors due to dietary factors, we enrolled only PCOS and healthy women with a low intake of folate (24). Because of the limitations and complexity of various approaches to evaluate food intake and the nutrients (79), we did not use any semiquantitative questionnaire or diet-quality scores, but only a measurement of serum folate. The cut-off established in the present study was very close, and it was lower in comparison to the one used by Somekawa *et al.* (<13.6 nmol/liter; Ref. 80). Thus, the differences in Hcy value were due probably only to genetic (*i.e.* C677T MTHFR mutation) or metabolic (*i.e.* PCOS) factors.

TABLE 4. Glucose and insulin levels at baseline and after OGTT in PCOS and control women according to MTHFR genotypes

	PCOS			Controls		
	CC	CT	TT	CC	CT	TT
Fasting						
Glucose (mmol/liter)	6.9 ± 3.2	6.9 ± 3.0	6.8 ± 2.9	5.6 ± 3.1	5.6 ± 3.0	5.5 ± 2.8
Insulin (μU/ml)	21.5 ± 6.9[a]	21.3 ± 6.7[a]	20.4 ± 6.8[a]	7.8 ± 2.1	7.9 ± 2.2	7.7 ± 2.0
OGTT						
$AUC_{glucose}$	1266 ± 516	1242 ± 569	1281 ± 572	1239 ± 479	1218 ± 466	1187 ± 450
$AUC_{insulin}$	7785 ± 1476[a]	7696 ± 1443[a]	7419 ± 1452[a]	2566 ± 388	2608 ± 414	2557 ± 371
$AUC_{glucose}/AUC_{insulin}$ ratio	0.20 ± 0.35[a]	0.18 ± 0.33[a]	0.19 ± 0.33[a]	0.52 ± 0.41	0.48 ± 0.39	0.47 ± 0.38

Data are expressed as mean ± SD.
[a] $P < 0.05$ *vs.* control group.

TABLE 5. Linear relation between Hcy levels and fasting glucose and insulin levels, $AUC_{glucose}$, $AUC_{insulin}$, and $AUC_{glucose}/AUC_{insulin}$ ratio after OGTT in PCOS and control women according to MTHFR genotypes

	PCOS (r)			Controls (r)		
	CC	CT	TT	CC	CT	TT
Fasting						
Glucose (mmol/liter)	0.28	0.25	0.26	0.24	0.20	0.23
Insulin (μU/ml)	0.42[a]	0.45[a]	0.10	0.41[a]	0.44[a]	0.18
OGTT						
$AUC_{glucose}$	0.32	0.27	0.27	0.28	0.25	0.26
$AUC_{insulin}$	0.61[a]	0.58[a]	0.19	0.46[a]	0.53[a]	0.23
$AUC_{glucose}/AUC_{insulin}$ ratio	0.64[a]	0.61[a]	0.21	0.60[a]	0.62[a]	0.25

[a] $P < 0.05$.

The exact significance of the absence of an association between MTHFR C677T mutation and serum Hcy levels in women with PCOS is unknown. It is possible to hypothesize that in PCOS women, the hormonal pattern (*i.e.* relative hyperestrogenism) may amplify the responsiveness of thermolabile MTHFR to plasma folate levels, inducing an absence of relationship between TT genotype and high Hcy levels in subjects with low folate intake. To explain the relationship between MTHFR C677T mutation and Hcy level in PCOS women, our research group is performing an evaluation of the effect of folate repletion or of a methionine load on Hcy concentrations in this subset of women. In fact, the use of methionine challenge reveals an additional 27% of patients with hyperhomocysteinemia and would have led to classification of more than 40% fewer subjects as being hyperhomocysteinemic (77, 81). Indeed, fasting and post-load elevations in Hcy had independent effects on the cardiovascular risk and had a multiplicative effect when present together. The relative risk for a subject with an elevated fasting Hcy was only 1.6 [95% confidence interval (CI), 1.0–2.2] and that for an elevated post-load was only 1.5 (95% CI, 1.0–2.2), whereas for subjects with both elevated fasting and post-load levels, the relative risk was 2.5 (95% CI, 1.7–3.5; Ref. 77).

Plasma levels of insulin seem to influence Hcy metabolism through effects on glomerular filtration or by influencing activity of pivotal enzymes in Hcy metabolism, as MTHFR and cystathione-β-synthase (CBS). In animal model, Fonseca *et al.* (82, 83) have shown that fasting plasma insulin correlated significantly and positively with plasma Hcy and MTHFR activity and negatively with CBS activity, supporting the hypothesis that insulin may regulate Hcy metabolism through regulation of the two hepatic key enzymes. The activity of the CBS and MTHFR was also affected when insulin and glucose were added to hepatocytes *in vitro* (84). More recently, it has been demonstrated that insulin has a direct role in the expression of CBS in the liver (85).

In normal weight, overweight, and obese premenopausal women, the Hcy levels are independently associated with insulin resistance (86), and this association seems to be stronger in hyperinsulinemic obese subjects (87). In a large observational study, Meigs *et al.* (88) confirmed that the fasting plasma Hcy levels are directly associated with insulin resistance.

Furthermore, the data regarding the relationship between insulin, insulin resistance, and Hcy levels are still contrasting (89–92). Metformin administration in women with PCOS can lead to the increase in Hcy levels (93). Gursu *et al.* (94) have shown that Hcy concentrations are decreased in experimentally induced insulin-dependent diabetes mellitus, and insulin administration normalizes Hcy levels in a dose-dependent manner.

In the present study, to evaluate the insulin resistance as a possible mechanism to explain the different Hcy concentrations in TT subjects with and without PCOS, $AUC_{glucose}$, $AUC_{insulin}$, and $AUC_{glucose}/AUC_{insulin}$ ratio were measured in each genotype group. Our data on the total study population with and without PCOS confirm that Hcy levels are directly related to insulin level after OGTT.

$AUC_{glucose}$, $AUC_{insulin}$, and $AUC_{glucose}/AUC_{insulin}$ ratio were significantly higher in the PCOS group than in the control group without differences within and between MTHFR genotypes. Moreover, the linear relation analysis between Hcy levels and $AUC_{glucose}$, $AUC_{insulin}$, and $AUC_{glucose}/AUC_{insulin}$ ratio showed a statistical significance only in CC and CT genotypes. In fact, in PCOS and control women, this significance was lost considering the TT genotype. In this view, our data suggest that the higher insulin resistance observed in PCOS women did not influence negatively the MTHFR activity. In addition, the relation between insulin resistance and Hcy levels in the different MTHFR genotypes could explain the difference in the results obtained in the different studies on this issue (86–94). Unfortunately, this remains unclear because in our study, subjects with TT genotype have Hcy levels unrelated to insulin resistance.

In conclusion, in the present study, we have demonstrated that the mean serum Hcy levels are not increased in women with PCOS and that the thermolabile MTHFR enzyme influences the serum Hcy concentrations only in healthy women, whereas it is not related to Hcy levels in PCOS. The

insulin resistance seems to play a role in Hcy metabolism only in women without thermolabile MTHFR enzyme.

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