Effects of metformin with or without supplementation with folate on homocysteine levels and vascular endothelium of women with polycystic ovary syndrome

Running Title: Metformin plus folate in patients with PCOS

Stefano Palomba\textsuperscript{a}, MD, Angela Falbo\textsuperscript{a}, MD, Francesco Giallauria\textsuperscript{b}, MD, Tiziana Russo\textsuperscript{a}, MD, Achille Tolino\textsuperscript{c}, MD, Fulvio Zullo\textsuperscript{a}, MD, PhD, Annamaria Colao\textsuperscript{d}, MD, PhD, Francesco Orio\textsuperscript{d,e}, MD, PhD.

\textsuperscript{a}Department of Obstetrics & Gynecology, University “Magna Graecia” of Catanzaro, \textsuperscript{b}Department of Clinical Medicine, Cardiovascular and Immunological Sciences, University “Federico II” of Naples; \textsuperscript{c}Department of Obstetrics & Gynecology, University “Federico II” of Naples; \textsuperscript{d}Department of Endocrinology, University “Federico II” of Naples, \textsuperscript{e}Endocrinology, University “Parthenope” of Naples, Italy

Address correspondence to: Stefano Palomba, MD
stefanopalomba@tin.it

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**Objective:** To evaluate whether the administration of metformin exerts any effects on serum homocysteine (Hcy) levels in patients with polycystic ovary syndrome (PCOS), and whether supplementation with folate enhances the positive effects of metformin on the structure and function of the vascular endothelium.

**Research Design and Methods:** Fifty patients affected by PCOS, without additional metabolic or cardiovascular diseases, were enrolled in a prospective, non-randomised placebo-controlled double-blind clinical study. They were grouped into two treatment arms that were matched for age and body mass index. Patients were treated with a six-month course of metformin (1,700 mg daily) plus folic acid (400 μg daily; experimental group, n=25) or placebo (control group, n=25). Complete hormonal and metabolic patterns, serum Hcy, folate, vitamin B12, endothelin-1 (ET-1) levels, brachial artery diameter at the baseline (BAD-B) and after reactive hyperaemia (BAD-RH), flow-mediated dilation (FMD), and intima–media thickness (IMT) in both common carotid arteries were evaluated.

**Results:** After treatment, a significant increase in serum Hcy levels was observed in the control group in comparison with the baseline values and the experimental group. A beneficial effect was observed in the concentrations of BAD-B, BAD-RH, FMD, IMT, and serum ET-1 in both groups. However, the results were improved more significantly in the experimental group than in the controls.

**Conclusion:** Metformin exerts a slight but significant deleterious effect on serum Hcy levels in patients with PCOS, and supplementation with folate is useful to increase the beneficial effect of metformin on the vascular endothelium.
Polycystic ovary syndrome (PCOS) is a heterogeneous disorder characterised by hyperandrogenism, ovarian dysfunction and polycystic ovarian morphology. Young patients with PCOS but no additional risk factors for cardiovascular disease have a significant impairment of endothelial structure and function (1). In particular, when compared with controls matched for age and body mass index (BMI), patients with PCOS had abnormal brachial artery diameter at baseline (BAD-B) and after reactive hyperaemia (BAD-RH), and showed abnormal flow-mediated dilation (FMD) and an increased intima–media thickness (IMT) in their common carotid arteries (1). In addition, plasma concentrations of endothelin-1 (ET-1), a biochemical marker of endothelial function, were altered significantly (1).

A subsequent clinical study showed that a six-month course of biguanidine metformin improved these parameters of endothelial structure and function significantly, which suggested that metformin has a beneficial effect in reducing the long-term risk of cardiovascular disease in patients with PCOS (2).

Moreover, in patients with type 2 diabetes mellitus, metformin has been demonstrated to increase serum levels of homocysteine (Hcy) (3-5). Increased concentrations of Hcy are a well-known risk factor for coronary heart disease and stroke (6). The acute effect of metformin on insulin sensitivity could either increase serum Hcy levels directly, or induce malabsorption of vitamin B12 indirectly (7). Other unknown adjunctive mechanisms cannot be excluded.

Based on these considerations, the aim of the present study was to evaluate whether the administration of metformin exerts any effect on serum Hcy levels in patients with PCOS, and whether supplementation with folate enhances the positive effects of metformin on the structure and function of the vascular endothelium.

RESEARCH DESIGN AND METHODS

The procedures used during the study were in accordance with the guidelines of the Helsinki Declaration on human experimentation and those of the Good Clinical Practice guidelines. The study was approved by the Institutional Review Boards of the “Magna Graecia” and “Federico II” Universities of Catanzaro and Naples, Italy, respectively. The study protocol was explained carefully to each woman, and written consent was obtained from each participant before entry into the study.

Patients: Between February 2004 and April 2006, 50 consecutive women with PCOS, who had been referred as outpatients to the two Academic Departments for the treatment of oligomenorrhoea, were enrolled in a prospective, non-randomised, placebo-controlled, double-blind clinical study.

The patients were allocated to one of two study groups and were matched on age and body mass index (BMI). Twenty-five participants (controls) received metformin and a placebo, whereas the remaining 25 patients (the experimental group) received the experimental treatment. The diagnosis of PCOS was based on the National Institute of Health (NIH) criteria (8).

The following general exclusion criteria were considered: age <18 or >35 years; BMI >35 kg/m²; neoplastic, metabolic, endocrine, hepatic, renal or cardiovascular disorders, or other concurrent medical illnesses; glucose intolerance; malabsorptive disorders; folate or vitamin B12 deficiency; current or previous (within the last six months) use of hormonal, anti-diabetic or anti-obesity drugs; intention to adopt a diet and/or a specific physical activity programme.
All participants had a normal level of physical activity and were non-smokers; none was known to abuse alcohol.

**Study protocol:** All participants (experimental and control groups) received metformin from the third day of a progesterone (P)-induced withdrawal bleeding, which involved the administration of 10 mg natural P intramuscularly (IM) at a starting dose of 850 mg (one tablet daily before lunch) for the first week. The dose rose to 1700 mg/day thereafter (two tablets daily, one before lunch and one before dinner).

The experimental group also received 400 \( \mu \text{g} \) of folic acid daily (one tablet/day), whereas the control group received an inert cellulose powder placebo (one tablet/day). Both folic acid and placebo tablets were taken in association with the metformin therapy. Patients were blinded to the treatment allocation (experimental or control group).

Throughout the study, no lifestyle modification was implemented; indeed, participants were instructed to follow their usual diet and physical activity. Each patient was also advised to use barrier contraception throughout the study.

At entry into the study and after six months of treatment, all participants underwent clinical and biochemical evaluations and a cardiovascular examination. All of the investigators involved in the study were blinded to the treatment allocation.

**Clinical Assessment:** Clinical assessment consisted of anthropometric measurements, which included height, weight, BMI (ratio between the weight and the square of the height) and waist-to-hip ratio (WHR, ratio between the waist and the circumference of the hip) and an assessment of the Ferriman–Gallwey score.

Measurements of heart rate (HR), systolic blood pressure (SBP) and diastolic BP (DBP) were also obtained. The SBP and DBP were measured in the right arm by standard methods with the participants in a relaxed sitting position, using a mercury sphygmomanometer. The average of six measurements taken by two independent examiners (each took three sets of measurements) was used.

Transvaginal ultrasonography was performed on each participant during the same baseline visit by an experienced operator (T.R.).

Daily physical activity was evaluated for each participant with the use of a leisure-time physical activity (LTPA) questionnaire and a calculation of the weekly energy expenditure score (total LTPA level) in metabolic equivalents per hour/week (METs-h/wk).

Diet and caloric intake were assessed by an experienced clinical dietician using a self-administered semi-quantitative validated food-frequency questionnaire and software designed for the analysis of food habits and the estimation of nutrient and caloric intake (WinFood, release 1.5; Medimatica, Martinsicuro, Teramo, Italy).

All participants were instructed to record in a personal daily diary the onset of any adverse events (AEs), specifying their characteristics (severity, duration, possible cause–effect relationship with drug administration), the characteristics of their menstrual cycles (length and quantity), the number of skipped tablets, and any changes in diet, physical activity or weight.

During the study, no monitoring of ovulation was performed by ultrasonography. However, ovulation was detected by a plasma P assay (> 10 ng/mL, SI: 32 nmol/L) performed seven days before the expected menses.

**Biochemical Assessment:** During the early proliferative phase (2\(^{nd}\)–3\(^{rd}\) day) of the P-induced uterine withdrawal bleeding (100 mg natural P IM), a venous blood
sample was taken from each participant between 8:00 am and 9:00 am after fasting for 12 hr and resting in bed, in order to assess a complete hormonal profile, lipid pattern, serum ET-1, glucose and insulin [at fasting and after a two-hour oral glucose tolerance test (OGTT)], Hcy, folate, and vitamin B12 levels.

In each participant, the homeostasis model of assessment (HOMA), the fasting glucose-to-insulin ratio (GIR) (mg/10^4 U), and the area under the curve (AUC) for glucose (AUC_{glucose}) and insulin (AUC_{insulin}), and the free androgen index (FAI) were calculated.

Serum levels of ET-1 were measured by an enzyme linked immunosorbent assay (ELISA) (Biomedica Gesellschaft, Wien, Austria) with a sensitivity of 0.05 pmol/litre, and intra- and inter-assay coefficients of variation (CV) of 4.5% and 6.9%, respectively (1, 2).

Serum Hcy levels were measured by high pressure liquid chromatography (HPLC) (9). The sensitivity of the assay for Hcy was greater than 0.25 µmol/litre of serum, and the inter- and intra-assay CV were 2% and 1%, respectively.

The levels of vitamin B12 and folate were analysed by capillary electrophoresis (P/ACE 5000 system, Beckman Coulter, Inc.) (9). The inter- and intra-assay CV for the vitamin B12 assay were 7.4% and 6.7%, respectively, and 4.1% and 4.2%, respectively, for the folate assay.

**Cardiovascular Studies:** All participants were studied during the same visit in a comfortable supine position in a quiet room, having fasted for at least 8–12 hr and been at rest for at least 4–6 h before the examination. Ambient light and temperature were controlled throughout the procedures (1, 2).

Brachial artery reactivity and the IMT of the common carotid arteries were studied using a colour Doppler ultrasound system by an experienced ultrasonographer (F.G.). Scans for the analysis of brachial reactivity were taken over the dominant brachial artery in a longitudinal orientation just proximal to the antecubital fossa using a high-resolution 7.5 MHz linear transducer. Blood flow to the limb was occluded by inflating a standard sphygmomanometry cuff on the upper arm (40 mmHg above SBP for 4 min) in order to induce ischaemia. The BAD-RH was measured at 30 sec, 1, 2, 3, and 4 min after the subsequent deflation of the cuff. In all studies, BP in the contralateral brachial artery was recorded at regular intervals, and the electrocardiogram was monitored continuously (1, 2).

Brachial artery FMD was calculated as the percentage change in BAD from baseline to 4 min after deflation of the cuff. In our studies, the intra- and inter-observer CV for the repeated measurements of the resting arterial diameter were 2.3 and 5.6%, respectively (1, 2).

Longitudinal ultrasonographic scans of the carotid artery were obtained using a high-resolution 10 MHz linear probe. All women were examined in the supine position with the head hyperextended and turned away from the side being scanned. Images were obtained from the distal portion of both common carotid arteries, 1cm–2 cm proximal to the carotid bulb and immediately proximal to the origin of the bifurcation (1, 2).

The IMT of the posterior wall of both common carotid arteries was measured as the distance between the junction of the lumen and intima and the junction of the media and adventitia at the end of diastole from the B-mode screen. The mean IMT for each side was calculated as the average of ten measurements made of the right and left carotid arteries using electronic calipers. In our studies, the intra- and inter-observer CV for the repeated measurements of IMT were 7.0% and 12.0%, respectively (1, 2).
**Statistical Analysis:** The sample size was calculated on the assumption that a difference of 0.1 mm in IMT is clinically relevant because it predicts a 10%–15% reduction in the risk of future myocardial infarction and a 13%–18% reduction in the risk of future stroke (10). Given that a mean IMT of 0.53 ± 0.09 [mm ± standard deviation (SD)] has been observed in patients with PCOS (1, 2), we needed to enroll at least 23 patients in each group to yield a statistically significant result with a power of 90%. To allow for an unpredictable number of withdrawals, we decided to enrol a total of 50 patients in the expectation that at least 23 patients would be left in each group. The criterion for significance (alpha) was set at 0.05. The test was two-tailed, which means that an effect in either direction would be interpreted. The power analysis and the sample size calculation were performed using SamplePower release 2.0 (SPSS Inc., Chicago, IL, US).

Considering the experimental study design and the expected drop-out rate, data were analysed by a per-protocol analysis on the basis of the treatment received and not on the treatment assignment. In fact, we decided to study the real effect of the treatments only in the subjects who took their allocated therapy.

For categorical variables, Pearson’s chi-squared test was performed; Fisher’s exact test was required when more than 20% of the expected values in the frequency tables were below 5.0.

The normal distribution of the data for continuous variables was evaluated with the Kolmogrov–Smirnov test. These data were expressed as means ± SD and analysed with an unpaired Student t test and the general linear model (GLM) for a repeated measures analysis, with the Bonferroni test for post hoc analysis as required.

Bivariate two-tailed correlations were performed when calculating the Spearman’s coefficient (Spearman's rho, \( r \)) and the significance of the correlation was set at the 0.05 level in order to study the relationships between the variations (\( \Delta \)) in Hcy levels (\( \Delta\text{-Hcy} \)), in insulin sensitivity indexes (\( \Delta\text{-HOMA, } \Delta\text{-GIR, } \Delta\text{-AUC}_{\text{insulin/AUC}_{\text{glucose}}} \)), and in IMT (\( \Delta\text{-IMT} \)).

Statistical significance was set for all analyses at \( p < 0.05 \). The Statistics Package for the Social Sciences (SPSS 14.0.1; SPSS Inc., Chicago, IL, US) was used for all statistical analyses.

**RESULTS**

All patients studied had a “full-blown” PCOS phenotype, which consisted of hyperandrogenism, oligo-anovulation and polycystic ovaries (PCO) (11). This was confirmed by the identification of PCO during transvaginal ultrasonography in all of the participants (11). During the study, the two treatments were tolerated well, and the total incidence of AEs was not significantly different between the two groups. The rate of withdrawals was similar in the two groups (two subjects in the experimental group and one in the control group). These three patients were excluded because they were not compliant with the treatment, and their data were not considered in the final analysis.

Table 1 shows the clinical, hormonal and metabolic data at the baseline and after six months of treatment in both groups. The proportion of lean (no patients in either group), normal-weight [7/23 (30.4%) vs. 9/24 (37.5%)], overweight [11/23 (47.8%) vs. 10/24 (41.7%)] and obese [5/23 (21.7%) vs. 5/24 (20.8%)] patients was not different between the groups (\( p = 0.756 \)).

Significant (\( p < 0.05 \)) changes were observed in both groups, without a difference between them with respect to the
levels of serum testosterone and SHBG and for the FAI compared with baseline values (Table 1). Similarly, a significant \( (p < 0.05) \) reduction in the levels of insulin, HOMA, AUC_{insulin}, AUC_{insulin/AUC_{glucose}} and LDL-C, and a significant increase in GIR, were detected after six months of treatment in both groups, with no difference between the two treatment arms (Table 1).

In the control group, serum Hcy levels were significantly \( (p < 0.05) \) higher after treatment than the baseline values, whereas no significant change from baseline was observed in the experimental group (Table 1). In addition, a significant \( (p < 0.05) \) difference in serum Hcy levels was detected between the groups after treatment (Table 1).

At the end of the study, no significant difference had been observed in the length, frequency or quantity of menstruation between the two groups (data not shown). In addition, no difference was found between groups with regard to the rate of ovulatory cycles \([89/138 (64.5\%) \text{ vs. } 90/144 (62.5\%); p = 0.728]\).

No further difference in any clinical, hormonal or metabolic parameter was observed between or within the experimental and control groups (Table 1).

Table 2 depicts the parameters of endothelial structure and function that were observed at baseline and after six months of treatment in the two groups. No significant difference in any endothelial parameter was observed between the two groups at baseline. After six months of treatment, a significant \( (p < 0.05) \) reduction in the levels of BAD-B, BAD-RH, IMT, and serum ET-1, and a significant \( (p < 0.05) \) increase in FMD were observed in the two groups in comparison with the baseline values (Table 2). In addition, significant \( (p < 0.05) \) differences between the two groups were detected for all these parameters (Table 2).

In both groups, no significant correlation was detected between \( \Delta \)-Hcy and \( \Delta \)-HOMA \( (r = -0.632, p = 0.233 \text{ and } r = -0.704, p = 0.412, \text{ for the experimental and control groups, respectively}) \), \( \Delta \)-GIR \( (r = 0.587, p = 0.189 \text{ and } r = 0.604, p = 0.242, \text{ for the experimental and control groups, respectively}) \), or \( \Delta \)-AUC_{insulin/AUC_{glucose}} \( (r = -0.765, p = 0.654 \text{ and } r = -0.678, p = 0.547, \text{ for the experimental and control groups, respectively}) \). Conversely, a significant correlation was observed between \( \Delta \)-Hcy and \( \Delta \)-IMT in both the experimental \( (r = -0.504; p = 0.042) \) and control \( (r = -0.632; p = 0.039) \) groups.

**CONCLUSIONS**

Administration of metformin exerted beneficial effects on the traditional cardiovascular risk factors and reduced the morbidity and mortality from cardiovascular events in patients with type 2 diabetes mellitus (12). However, metformin also reduced serum levels of folate and vitamin B12 and increased serum Hcy levels in patients with diabetes (13, 14), even after only a short period (5). Long-term administration of metformin was shown to result in malabsorption of vitamin B12 (15), although the correlation between serum Hcy levels and absorption of folate or vitamin B12 was not clear. Unexpectedly, the improved sensitivity to insulin associated with metformin may increase plasma Hcy concentrations in obese patients with diabetes (6) or without diabetes (16).

Unlike the levels of serum Hcy in patients with diabetes, the response of serum Hcy levels to the administration of metformin in women affected by PCOS was unclear, probably because of the heterogeneity of the studied populations (17-19). In agreement with previous data (17, 19), the results of the study described herein showed a significant increase in serum Hcy levels after six months of metformin
Metformin plus folate in patients with PCOS

treatment in a heterogeneous population with PCOS. Our findings were not confirmed by Yilmaz et al. (18), who observed that there was no change in the concentration of plasma Hcy after the administration of metformin in lean patients with PCOS. However, even if the cohort studied in the latter trial was comprised of a well-selected sample, they did not represent the broad spectrum of patients who have PCOS.

Our findings demonstrated that the increase in plasma Hcy concentrations that was attributable to metformin treatment was not associated with any significant effect on the level of serum folate or vitamin B12. In addition, no significant relationship was detected between Δ-Hcy and changes in the indexes of insulin sensitivity, including Δ-HOMA, Δ-GIR, and Δ-AUC_{insulin}/AUC_{glucose}. These data suggest that the increase in plasma Hcy can be explained by factors that act in addition to metformin treatment to improve insulin sensitivity. In fact, the administration of rosiglitazone, which is an insulin-sensitising drug that improves insulin sensitivity more significantly than metformin, resulted in the opposite physiological effect to metformin because it decreased serum Hcy levels (5).

In agreement with our previous data (1, 2), the current study confirmed the beneficial effect of metformin on the structure and function of the vascular endothelium in young patients with PCOS. After six months of treatment, BAD-B, BAD-RH, IMT and serum ET-1 levels were significantly lower than the baseline values, whereas a significant increase in FMD was detected.

The results of folic acid supplementation in patients with PCOS who also received metformin were very interesting. A previous under-powered randomised controlled study (20) showed that serum Hcy levels were reduced by 21.2% after the administration of folic acid and by 8.3% after the administration of B-group vitamins in patients affected by PCOS who had also been treated for 12 weeks with metformin. Our results confirmed the significant reduction in serum Hcy levels after supplementation of metformin treatment with 400 µg of folic acid daily, in comparison with those who had received a placebo. This represents a potential beneficial effect in terms of reducing the incidence of long-term adverse cardiovascular events in patients with PCOS.

The effects of a reduction in serum Hcy levels on the vascular endothelium in patients affected by PCOS have not been demonstrated previously. In the current study, we reported a significant correlation between the variation in Hcy levels and changes in the IMT after treatment with metformin and folic acid.

To our knowledge, this is the first study to investigate the effects of supplementation with folate in patients with PCOS who are being treated with metformin. After six months of metformin treatment with supplementation with folic acid, a significant improvement was observed in all the markers of structure and function of the vascular endothelium. More interestingly, the extent of improvement in the structure and function of the endothelium was significantly different between patients who received supplementation with folic acid and those who received the placebo. In fact, significant differences were observed in the values for BAD-B, BAD-RH, FMD, IMT and serum ET-1.

The mechanisms that underlie these beneficial effects related to supplementation of metformin treatment with folic acid are not understood completely. In fact, the well known physiological effect of folic acid is a reduction in the level of Hcy in serum.
However, its effects may also involve other unknown mechanisms. In this regard, folic acid has been shown in several clinical studies to improve endothelial function independently of the reduction of Hcy levels (21, 22). Thus, although serum folate levels showed a non-significant increase in the current study, a direct effect of folic acid on the endothelium cannot be excluded and could explain, at least partially, our findings.

On the other hand, no beneficial or adverse effects were reported on the incidence of major cardiovascular events following supplementation with folic acid and vitamin B complex in a high-risk population of women over 7.3 years of follow-up (23). A recent study (24) showed that long-term treatment with B vitamins in an attempt to reduce the level of Hcy did not reduce IMT or increase FMD in patients who had suffered strokes, even though a modest increase in FMD without any improvement in the structure of the vascular endothelium was observed after short-term treatment with B vitamins. Conversely, treatment of patients affected by type 2 diabetes mellitus with high doses of metformin and folic acid to reduce serum Hcy levels resulted in improved elasticity of small arteries, which suggested that folic acid has an additional beneficial effect on the vascular system (14). This effect was also observed in the current study of patients with PCOS.

Our findings in women affected by PCOS may have clinical implications. In fact, the power analysis was based on previous data from a meta-analysis, which showed a hazard risk for myocardial infarction and stroke of up to 1.15 [95% confidence interval (CI): 1.12–1.17] and 1.18 (95% CI: 1.16–1.21), respectively, for a reduction of 0.1 mm in IMT in the general population (10). In this regard, we consider that a difference in IMT greater than 0.1 mm between experimental and control treatments is most likely to be related to a clinically significant reduction of long-term risk, although this can only be proven over a long-term follow-up.

Finally, there are several limitations to the current study that warrant consideration. Firstly, this is a preliminary clinical study conducted on a heterogeneous Italian population with PCOS. Secondly, to define any short- and long-term results that are significant clinically, patients should be treated and followed for a longer period of time. Thirdly, further studies are needed before a clear indication can be obtained of the dose and regimen of supplementation with folic acid that is required to optimise the vascular benefits of the administration of metformin.

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### Table 1. Clinical, hormonal and metabolic data in PCOS patients treated with metformin plus supplementation with folate (experimental group) or metformin plus placebo (control group) at study entry and after six months of treatment.

| Group          | Experimental (n = 23) | Control (n = 24) |
|----------------|----------------------|------------------|
|                | Baseline       | Six months     | Baseline       | Six months     |
| Age (yr)       | 26.9±3.1       | 27.0±2.8       | 26.4±3.1       | 27.9±3.4       |
| BMI (Kg/m²)    | 27.9±2.6       | 27.8±2.9       | 28.1±3.1       | 27.9±3.4       |
| WHR            | 0.82±0.3       | 0.82±0.4       | 0.84±0.5       | 0.83±0.6       |
| Ferriman-Gallwey score | 11.6±3.2   | 10.8±3.7       | 11.9±2.8       | 11.4±3.3       |
| LTPA           | 8.3±3.4        | 8.3±3.4        | 8.3±3.4        | 8.7±3.5        |
| HR (beats/min) | 71.9±4.3       | 71.5±5.7       | 72.3±6.1       | 72.0±6.4       |
| SBP (mmHg)     | 119.6±10.8     | 114.6±14.2     | 116.5±13.1     | 113.2±14.3     |
| DBP (mmHg)     | 70.7±9.8       | 68.7±6.5       | 71.5±6.9       | 71.3±7.2       |
| FSH (mIU/mL)   | 5.8±1.3        | 6.3±1.6        | 5.8±1.5        | 5.9±1.8        |
| LH (mIU/mL)    | 16.1±4.4       | 16.7±4.2       | 15.3±5.4       | 16.2±6.2       |
| TSH (µU/mL)    | 2.7±0.9        | 2.1±1.1        | 2.6±1.3        | 2.6±2.3        |
| PRL (ng/mL)    | 9.0±2.0        | 8.4±2.1        | 7.9±2.3        | 8.2±2.7        |
| E₂ (pg/mL)     | 40.8±4.8       | 41.5±4.3       | 38.2±4.4       | 39.5±6.4       |
| P (ng/mL)      | 0.8±0.1        | 0.7±0.2        | 0.8±0.3        | 0.8±0.4        |
| 17-OHP (µg/L)  | 0.7±0.3        | 0.7±0.5        | 0.8±0.1        | 0.7±0.2        |
| T (ng/mL)      | 1.5±0.4        | 1.1±0.3        | 1.6±0.3        | 1.3±0.4        |
| A (ng/mL)      | 1.7±0.4        | 1.6±0.5        | 1.7±0.2        | 1.6±0.4        |
| DHEAS (ng/mL)  | 2726.6±294.8   | 2700.3±257.9   | 2689.9±256.9   | 2602.3±262.4   |
| SHBG (nmol/L)  | 27.5±5.6       | 38.2±6.3*      | 28.4±6.9       | 40.2±7.3*      |
| FAI (%)        | 20.0±6.1       | 10.4±3.9*      | 21.2±7.1       | 12.0±5.2*      |
| Fasting glucose (mg/dL) | 84.7±9.0 | 82.6±7.2 | 79.3±10.4 | 80.3±11.2 |
| Fasting insulin (µU/mL) | 14.6±5.3 | 10.2±4.0* | 15.1±4.8 | 10.9±4.3* |
| GIR (mg/10⁴U)  | 8.9±5.2        | 11.1±6.6*      | 7.6±6.1        | 11.3±7.0*      |
| HOMA           | 5.4±2.9        | 5.1±1.7*       | 4.9±3.7        | 4.2±3.1*       |
| OGTT           | 1182.5±143.9   | 1130.0±148.3   | 1222.7±128.2   | 1246.9±135.9   |
| AUCglucose/AUCinsulin ratio | 0.32±0.12 | 0.20±0.06* | 0.37±0.01 | 0.21±0.11* |
| TC (mg/dL)     | 150.8±42.5     | 146.7±38.7     | 143.1±30.9     | 141.9±43.2     |
| HDL-C (mg/dL)  | 85.1±19.3      | 86.7±15.5      | 90.8±16.9      | 91.2±17.3      |
| LDL-C (mg/dL)  | 73.5±19.4      | 66.6±18.7*     | 74.8±23.9      | 68.6±27.1*     |
| TG (mg/dL)     | 132.9±26.6     | 124.0±26.6     | 123.6±26.7     | 126.4±27.6     |
| Vitamin B12 (ng/mL) | 379.3±108.1 | 384.9±118.3 | 379.3±108.1 | 364.7±123.4 |
| Folate (nmol/mL) | 8.5±2.9     | 8.9±3.1        | 8.6±2.4        | 8.2±3.2        |
| Hcy (µmol/L)   | 10.5±3.9       | 9.7±4.2        | 11.1±4.4       | 13.9±3.8**     |

Data expressed as mean± standard deviation (SD) or as number and percentage. *p < 0.05 vs. baseline. °p < 0.05 vs. experimental group. The LTPA level was graded into four categories: (a) no LTPA; (b) light LTPA most of the week; (c) moderate LTPA (large increase in heart rate, breathing and perspiration) for at least 20 min once or twice a week; and (d) strenuous LTPA for at least 20 min three times a week or more; A: androstenedione; AUC: area under the curve; BMI: body mass index; DBP: diastolic blood pressure; DHEAS: dehydroepiandrosterone sulfate; E₂: 17β-estradiol; FAI: free androgen index; FSH: follicle-stimulating hormone; GIR: glucose-to-insulin ratio; Hcy: homocysteine; HDL-C: high-density lipoprotein-cholesterol; HOMA: homeostasis model of assessment; HR: heart rate; LDL-C: low-density lipoprotein-cholesterol; LH: luteinizing hormone; LTPA: leisure-time physical activity; OGTT: oral glucose tolerance test; 17-OHP: 17-OH-progesterone; P: progesterone; PRL: prolactin; SBP: systolic
Metformin plus folate in patients with PCOS

blood pressure; SHBG: sex-hormone binding globulin; T: total testosterone; TC: total cholesterol; TG: triglycerides; TSH: thyroid-stimulating hormone; WHR: waist-to-hip ratio.

Table 2. Endothelial parameters in PCOS patients treated with metformin plus supplementation with folate (experimental group) or metformin plus placebo (control group) at study entry and after six months of treatment.

| Group      | Experimental (n = 23) | Control (n = 24) |
|------------|----------------------|------------------|
|            | Baseline             | Six months       | Baseline             | Six months       |
| BAD-B (mm) | 3.4±0.5              | 2.8±0.6*         | 3.5±0.6              | 3.0±0.5*°        |
| BAD-RH (mm)| 3.7±0.4              | 3.4±0.6*         | 3.7±0.3              | 3.6±0.7*°        |
| FMD (%)    | 14.4±1.9             | 16.2±2.1*        | 14.4±2.0             | 15.3±1.8*°       |
| IMT (mm)   | 0.53±0.09            | 0.39±0.08*       | 0.55±0.11            | 0.50±0.07*°      |
| ET-1       | 1.1±0.4              | 0.7±0.5*         | 1.2±0.6              | 0.9±0.5*°        |

Data expressed as mean ± standard deviation (SD). *p < 0.05 vs. baseline. °p < 0.05 vs. experimental group. BAD-B: brachial artery diameter at baseline; BAD-RH: brachial artery diameter after reactive hyperaemia; FMD: flow-mediated dilation; IMT: intima–media thickness; ET-1: endothelin-1.