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Administration of B-group vitamins reduces circulating homocysteine in polycystic ovarian syndrome patients treated with metformin: a randomized trial

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BACKGROUND: The aim of the current study was to assess the effects of B-group vitamins and folic acid administration on serum levels of homocysteine (Hcy) in patients with polycystic ovarian syndrome (PCOS) on short-term metformin treatment. METHODS: Patients were randomly assigned to one of three treatment groups. Group 1 patients ($n = 20$) received metformin (850 mg twice daily); group 2 patients ($n = 20$) received metformin (850 mg twice daily) and B-group vitamins (vitamin B_1, 250 mg; vitamin B_6, 250 mg; vitamin B_{12}, 1000 μg twice daily); and group 3 patients ($n = 20$) received metformin (850 mg twice daily) and folic acid (174 μg twice daily). In all groups, lipid profiles and plasma total Hcy, vitamin B_{12}, folic acid and glucose levels were recorded at baseline and at 3 months. RESULTS: A 26.5% increase in Hcy levels was seen after 12 weeks of metformin therapy, while 21.17 and 8.33% decreases in Hcy levels were detected when B-group vitamins or folic acid plus metformin were given respectively. There were no statistically significant differences recorded in insulin sensitivity using homeostasis model assessment in the three groups. CONCLUSION: These findings suggest that B-group vitamins and folic acid administration counteract the Hcy-increasing effect seen with metformin therapy.

Key words: B-group vitamin/folic acid/hyperhomocysteinaemia/metformin/PCOS

Introduction

Increased total plasma homocysteine (Hcy) is an accepted risk factor for premature cardiovascular disease (CVD) and stroke risk in healthy populations (Homocysteine Studies Collaboration, 2002). Experimental studies have demonstrated that high plasma concentrations of Hcy may cause vascular damage and alteration in the coagulation process (Audelin and Genest, 2001). Ten per cent of the risk of coronary artery disease in the general population is attributable to an increase in Hcy levels (Boushey *et al.*, 1995). They reported that an increase of 5 μmol/l in plasma Hcy levels enhances the risk for CVD by 1.6–1.8-fold. Plasma Hcy levels have been shown to correlate with blood pressure (Hoogeveen *et al.*, 1998), and increased levels of Hcy have been positively associated with insulin levels (Fonseca *et al.*, 2000; McCarty, 2000).

Polycystic ovarian syndrome (PCOS) is one of the most common endocrinopathies, affecting 3–7% of women of reproductive age (Knochenhauer *et al.*, 1998; Asuncion *et al.*, 2000). It is not known whether women with PCOS have a greater risk of cardiovascular disease compared with appropriately aged controls (Legro *et al.*, 2001). Although epidemiological studies have not shown an increased incidence of death from cardiovascular events in women with PCOS (Pierpoint *et al.*, 1998), all current data suggest that PCOS possesses the intrinsic conditions that lead to an increased incidence of factors predisposing to cardiovascular diseases (Loverro, 2004). More than 30% of lean and 75% of obese women with PCOS have hyperinsulinaemia (Conway *et al.*, 1992). Insulin resistance, dyslipidaemia, impaired glucose tolerance, type 2 diabetes mellitus, and elevated systolic blood pressures are more prevalent in obese young women with PCOS, which suggests that women with PCOS are at an increased risk of CVD (Wild *et al.*, 1985; Wild, 2002a; Conway *et al.*, 1992; Rajkhowa *et al.*, 1994; Guzick, 1996; Dunaif, 1997). Cross-sectional studies assessing prevalence suggest that women with PCOS are at greater risk for premature development of diabetes mellitus (Wild, 2002b). Studies on high blood pressure and dyslipidaemia in women with PCOS patients to date offer conflicting results. Sampson *et al.*, (1996) found no differences in 24 h, daytime and night-time ambulatory blood pressure measurements. However, Holte *et al.*, (1996) have reported higher daytime systolic and mean ambulatory arterial blood pressures. A significant increase in left ventricular mass index and a diastolic filling have been described in women of normal weight with PCOS by Orio

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et al., (2004). On the other hand, Zimmermann *et al.*, (1992) suggest that despite profound insulin resistance and hyperinsulinaemia, women with PCOS do not have increased arterial pressure or left ventricular mass. Women with PCOS appear to have lower high-density lipoprotein (HDL) cholesterol and higher triglyceride (TG) levels independent of body weight (Wild, 2002a). Most, but not all, studies report this profile. Legro *et al.*, (2001) have reported that elevations in low-density lipoprotein cholesterol (LDL-C) were the predominant lipid abnormality when obese women with PCOS had relatively elevated HDL-C levels. Talbott *et al.*, (1998) suggest that LDL-C is substantially higher in young women with PCOS than in matched controls, while pre- to peri-menopausal women (aged ≥ 40 years) with PCOS have LDL-C and total cholesterol levels similar to those of age-matched controls. Additionally, significantly higher serum Hcy concentrations have been found in women with PCOS compared with controls by several authors (Yarali *et al.*, 2001; Loverro *et al.*, 2002; Schachter *et al.*, 2003; Wijeyaratne *et al.*, 2004), while others have found no association between PCOS and Hcy (Sills *et al.*, 2001; Orio *et al.*, 2003). Women with PCOS appear to be at increased risk of CVD when markers for atherosclerosis, such as intima-media thickness of femoral and carotid arteries (Talbott *et al.*, 2000; Lakhani *et al.*, 2004), are examined. Moreover, an increased prevalence of coronary artery (Christian *et al.*, 2003; Talbott *et al.*, 2004) and aortic calcification recently has been demonstrated among middle-aged women with PCOS (Talbott *et al.*, 2004) when compared with controls. Whether PCOS is an independent risk for circulatory death remains unclear, but current literature indicates that women with PCOS cluster risk factors for premature morbidity and mortality (Wild, 2002b).

Recognition of insulin resistance as a principal factor in the pathogenesis of PCOS has led to the use of insulin sensitizers for its treatment (Seli and Duleba, 2002). In the treatment of PCOS, the most extensively studied insulin sensitizer is metformin—an oral antihyperglycaemic agent used initially in the treatment of type 2 diabetes mellitus. It has been shown that metformin increases total serum Hcy levels in non-diabetic male patients with coronary heart disease (Carlsen *et al.*, 1997), and adding metformin to insulin therapy in type 2 diabetes mellitus reduces levels of folic acid and vitamin B_{12}, which results in a modest increase in Hcy levels within 12 weeks (Wulffele *et al.*, 2003). The Hcy-increasing effect of metformin in patients with PCOS has been demonstrated by Vrbikova *et al.* (2002). Recently, we demonstrated that 3 months of metformin and rosiglitazone therapy resulted in a significant increase in plasma Hcy concentrations in patients with PCOS (Kilicdag *et al.*, 2005).

Previously, folic acid administration has been shown to reduce Hcy levels in healthy subjects (Brattström *et al.*, 1988; Ubbink *et al.*, 1993), patients with kidney disease (Arnadottir *et al.*, 1993), and in patients with vascular disease (Brattström *et al.*, 1990; Landgren *et al.*, 1995). Furthermore, in patients with non-insulin-dependent diabetes mellitus on long-term metformin treatment, the Hcy-increasing effect of metformin has been shown to be counteracted by folate administration (Aarsand and Carlsen, 1998).

Folic acid, vitamin B_6 and vitamin B_{12} are all co-factors in Hcy metabolism. Serum vitamin B_{12} and folic acid levels are known to decrease during metformin therapy. Hence, during metformin therapy, Hcy levels might increase. In view of these considerations, we studied the effects of B-group vitamins and folic acid administration on serum levels of Hcy in patients with PCOS on short-term metformin treatment.

Materials and methods

This study was conducted in the Department of Obstetrics and Gynecology of Baskent University School of Medicine, between August 2003 and August 2004. Sixty women with PCOS participated in this prospective, randomized study. The study was approved by the Ethical Committee of Baskent University. Informed consent was obtained from each patient just before entering the study.

Patients with oligomenorrhoea (cycle intervals >35 days), amenorrhoea, anovulatory infertility, or a hirsutism score of >7 according to Ferriman and Gallway (1961), who also had at least one of the following criteria—an elevated serum testosterone level (>0.82 ng/ml) or appearance of PCO—were diagnosed as having PCOS, after all other causes of hyperandrogenism had been excluded (Homburg, 2002). Sonographic diagnosis of PCO was confirmed if ≥ 10 subcapsular follicular cysts, 2–8 mm in diameter, arranged around a thickened ovarian stroma were apparent (Adams *et al.*, 1986). All patients with elevated 17OH-progesterone levels were evaluated for 21-hydroxylase deficiency; none of the patients had 21-hydroxylase deficiency. Subjects treated with hormonal medications within the prior 3 months also were excluded from the study. When evaluating inclusion criteria according to 'Rotterdam 2003' criteria for PCOS (not published at the start of the study: Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004), we found that all patients fulfilled them, that is, at least two of the three criteria: polycystic ovaries, clinical and/or biochemical signs of hyperandrogenism, and oligo- and/or anovulation.

Patients were randomly assigned to one of three treatment groups. The patients were randomized into three groups by an allocation sequence generated from a random number table and assigned through consecutively numbered, opaque, sealed envelopes. Patients in group 1 ($n = 20$) received metformin (850 mg twice daily) (Glucophage®; Merck, Turkey); patients in group 2 ($n = 20$) received metformin (850 mg twice daily) and B-group vitamins [vitamin B_1 (250 mg); vitamin B_6 (250 mg); vitamin B_{12} (1000 μg) twice daily (Apikobal®; Santa Farma, Turkey)]; and patients in group 3 ($n = 20$) received metformin (850 mg twice daily) and folic acid [folic acid (174 μg), vitamin D (1200 μg) and calcium (666.670 mg) twice daily (Folic Plus®; Assos, UK)] for 3 months. Sixty patients were included, and 49 patients completed the study. Three patients became pregnant (two in the metformin group and one in the metformin plus folic acid group). Three patients withdrew because of gastrointestinal adverse effects of metformin (two in the metformin group and one in the metformin plus folic acid group). Three patients withdrew from the study owing to lack of motivation, and another two patients left the study without giving any reason. Fourteen patients in the metformin therapy group, 17 patients in the metformin plus folic acid therapy group, and 18 patients in the metformin plus B-group vitamins therapy group completed the study (Figure 1).

Hormonal parameters [FSH, LH, estradiol (E_2), total testosterone, free testosterone, dehydroepiandrosterone sulphate (DHEAS) and prolactin (PRL)], lipid profile [total cholesterol (total-C),

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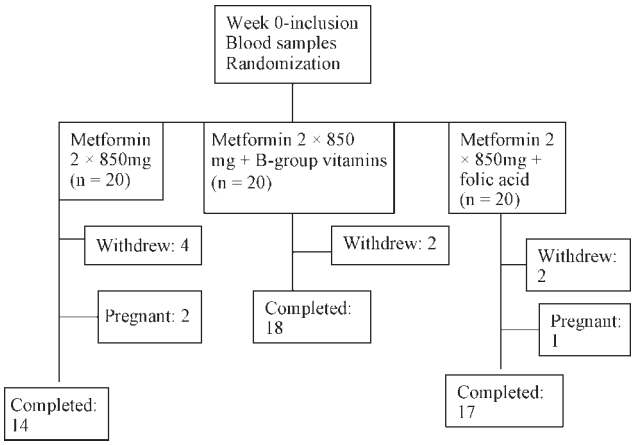

Figure 1. Chart of patients who were randomized, progressing through the trial of metformin, metformin plus B-group vitamins or metaformin plus folic acid.

high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), very low-density lipoprotein cholesterol (VLDL-C), triglyceride (TG), lipoprotein (Lp) (a), apolipoprotein (Apo) A_1, and Apo B)], Hcy concentrations, vitamin B_{12}, folic acid, and basal insulin levels were assessed, and a 75 g oral glucose tolerance test was performed before and after treatment in all patients. Blood samples were obtained on the third day of the menstrual cycle or when a spontaneous luteal phase was excluded by serum progesterone measurements (serum progesterone measurements <3 ng/ml). Ovulation was defined as serum progesterone measurements >3 ng/ml on the 21st day of the menstrual cycle.

Samples were collected at 08:00 (12 h fasting state) and at 120 min after glucose ingestion. Plasma glucose levels were measured using the glucose oxidase method; plasma insulin concentrations were measured by the microparticle enzyme immunoassay method (AxSYM insulin assay; Abbott, Japan).

Hcy levels are influenced by several variables, including smoking, renal function, vitamin B status, and enzyme dysfunction states. Renal status was examined before the women entered the study. All patients had normal creatinine levels. Vitamin B_{12} and folate levels were examined before and after treatment. All women were non-smokers. Patients with folic acid and vitamin B_{12} deficiencies were excluded from the study. None of the patients had used metformin or any type of vitamin before initiation of the trial.

Levels of plasma fasting glucose, total-C, HDL-C and TG were determined by the colorimetric method using a Cobas Mira Plus autoanalyser (Roche Diagnostics, Germany). LDL-C and VLDL-C levels were calculated by the formula of Friedwald (1972). Apo A_1, Apo B, and Lp (a) were quantified by the immunoturbidimetric method in a Roche/Hitachi 912 autoanalyser (DP, modular system, Japan). Insulin, LH, FSH, E_2, PRL and Hcy concentrations were measured using an AxSYM hormone autoanalyser (Abbott Laboratories, USA) using the microparticle enzyme immunoassay method. Total testosterone and DHEAS were measured in an Immulite One autoanalyser (Bio Diagnostic Products Corp., USA) using the chemiluminescent method. Insulin sensitivity was calculated using the homeostasis model assessment (HOMA) [(formula: fasting glucose (mmol/l) $\times$ fasting insulin (μIU/ml)/22.5)] (Matthews *et al.*, 1985). Intra- and inter-assay CV for glucose were 0.4 and 1.2%, 0.6 and 1.3%, and 0.7 and 1.1%, at 82.2, 81.4 and 80.5 mg/dl respectively. Intra- and inter-assay CV of the insulin were 2.6 and 1.8%, 4.1 and 2.5%, and 2.9 and 2%, at 8.7, 42.2 and 126.2 mIU/ml respectively.

Hcy, mixed disulphide, and protein-bound forms of Hcy in the samples were reduced to form free Hcy using dithiothreitol. Free Hcy was converted to *S*-adenosyl-L-Hcy (SAH) using SAH hydrolase and excess adenosine. SAH and labelled fluorescein tracer compete for sites on the monoclonal antibody molecule. The intensity of polarized fluorescent light was measured using a fluorescence-polarization immunoassay (FPIA) technique optical unit. The coefficient of variation of FPIA was 4.6% at 7.99 μmol/l, 3.1% at 13.71 μmol/l, and 2.8% at 26.67 μmol/l.

Plasma folic acid and vitamin B_{12} concentrations were measured using the chemiluminescent method with an E170 immunoassay analyser (Roche Diagnostics Corp., USA). The electrochemiluminescence immunoassay (ECLIA) was used on a Roche Modular Analytics E170 immunoassay analyser (Roche Diagnostics).

The primary outcomes were changes in Hcy, folic acid and vitamin B_{12} levels. Secondary outcomes included changes in body mass index (BMI), lipid profile, HOMA levels and menstrual cycle pattern with metformin, metformin plus B-group vitamins, and metformin plus folate therapy.

Statistical analyses

Sample size calculations, assuming 80% power to detect 2 μmol/l changes in Hcy levels between groups, indicated the need for 15 patients in each group.

Baseline characteristics of the three groups were compared using a one-way analysis of variance (ANOVA). Repeated measures ANOVA were used to analyse changes in variables before and after treatment in all groups. Repeated measures ANOVA was also used to assess significance within and between groups. The Bonferroni *post hoc* test was applied. Data are expressed as mean $\pm$ SEM; $P < 0.05$ was considered statistically significant. All statistical procedures were performed using SPSS software (Statistical Package for the Social Sciences, version 10.0; SSPS Inc., USA).

Results

There were no statistically significant differences in baseline age, BMI, parity, waist:hip ratio, Hcy concentrations, or vitamin B_{12} or folic acid levels between the groups (Table I). There were no statistically significant differences in hormone levels [FSH, LH, E_2, total testosterone, free testosterone, DHEAS, and PRL] or lipid profile [HDL-C, total-C, LDL-C, VLDL-C, TG, Lp(a), Apo A1, and Apo B] either.

Table I. Baseline characteristics of the patients according to treatment group (mean $\pm$ SEM)

	Group 1 ($n = 14$)	Group 2 ($n = 18$)	Group 3 ($n = 17$)
Age (years)	24.14 $\pm$ 6.92	22.00 $\pm$ 3.73	24.94 $\pm$ 6.67
Parity	0.21 $\pm$ 0.43	0.22 $\pm$ 0.55	0.65 $\pm$ 1.06
Body mass index (kg/m^2)	26.02 $\pm$ 5.98	25.24 $\pm$ 5.09	28.58 $\pm$ 5.43
Waist:hip ratio	0.80 $\pm$ 0.65	0.79 $\pm$ 0.06	0.80 $\pm$ 0.07
Homocysteine (μmol/l)	9.56 $\pm$ 0.81	11.43 $\pm$ 0.62	11.96 $\pm$ 0.81
Folic acid (ng/ml)	8.32 $\pm$ 0.58	6.86 $\pm$ 0.71	8.03 $\pm$ 0.73
Vitamin B_{12} (pg/ml)	281.83 $\pm$ 24.51	236.27 $\pm$ 42.39	219.05 $\pm$ 23.32

There were no significant differences between groups.
Group 1 = metformin; Group 2 = metformin plus vitamin B_{12}; Group 3 = metformin plus folic acid.

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Table II. Baseline hormonal characteristics of the patients according to treatment group (mean ± SEM)

	Group 1 ($n = 14$)	Group 2 ($n = 18$)	Group 3 ($n = 17$)
FSH (mIU/ml)	6.55 ± 0.43	5.27 ± 0.32	4.75 ± 0.35
LH (mIU/ml)	13.31 ± 3.58	9.76 ± 1.08	11.34 ± 2.36
Total testosterone (ng/ml)	0.68 ± 0.11	0.77 ± 0.99	0.81 ± 0.09
Free testosterone (pg/ml)	2.35 ± 0.18	2.81 ± 0.35	3.03 ± 0.26
17OH-progesterone (ng/ml)	1.10 ± 0.20	1.29 ± 0.24	1.09 ± 0.16
DHEAS (ng/ml)	2859.45 ± 442.88	2586.10 ± 283.06	2592.67 ± 270.93

Group 1 = metformin; Group 2 = metformin plus vitamin B_{12}; Group 3 = metformin plus folic acid.
DHEAS = dehydroepiandrosterone sulphate.

All of the women in the study had PCO appearance on sonography. Eleven of 14, 14 of 18, and 13 of 17 patients had oligomenorrhoea or amenorrhoea in groups 1, 2 and 3 respectively. Seven of 14 patients (50%) in group 1, six of 18 patients (33.3%) in group 2, and seven of 17 patients (41.2%) in group 3 had clinical and biochemical signs of hyperandrogenism. Six of 14, four of 18, and five of 17 patients had clinical hirsutism in groups 1, 2 and 3 respectively. Baseline hormonal characteristics of the patients are given in Table II.

Hcy and vitamin B assessments

Hcy levels increased from 9.56 ± 0.81 to 11.97 ± 1.01 μmol/l ($P < 0.001$) in group 1. However, Hcy levels decreased from 11.43 ± 0.62 to 8.66 ± 0.49 μmol/l ($P < 0.001$) and from 11.96 ± 0.81 to 10.71 ± 0.70 μmol/l ($P = 0.04$) in groups 2 and 3 respectively. Folic acid levels increased only in group 3, from 8.03 ± 0.73 to 13.30 ± 1.93 ng/ml ($P = 0.02$). Vitamin B_{12} levels increased only in group 2, from 236.27 ± 42.39 to 486.55 ± 66.26 pg/ml ($P = 0.004$) (Table III). When Hcy, folic acid and vitamin B_{12} were compared between the three groups after treatment, significant differences were found only in group 2 as compared to group 3 with respect to folic acid ($P = 0.007$) and vitamin B_{12} ($P = 0.014$).

Glucose and lipid metabolism and body weight assessments

There were no statistically significant differences recorded in HOMA levels between the three groups. No changes were recorded in the HDL-C, total-C, LDL-C, VLDL-C, TG, Lp (a), and Apo B levels in the three groups. Apo A_1 levels decreased from 147.50 ± 9.21 to 121.25 ± 10.79 mg/dl ($P = 0.04$) in group 2. Waist:hip ratio and weight were not altered with treatment (Table IV). No significant differences in HOMA, HDL-C, LDL-C, VLDL-C, TG, ApoA1, ApoB, Lp(a), and waist:hip ratio were found between groups after treatment. There was significant difference only between groups 2 and 3 with respect to weight after treatment ($P = 0.026$).

Ovulation and menstrual pattern

After treatment with metformin, seven of 49 (14.2 %) patients ovulated, and 27 of 49 (55.1%) patients had regular menses.

Adverse effects

Seven of the 60 patients on metformin had problems with nausea and vomiting; three patients discontinued therapy owing to nausea and vomiting. At the end of therapy, four of 49 patients complained of gastrointestinal adverse effects, two patients complained of headache, and two patients complained of fatigue.

Discussion

The present study shows that serum levels of Hcy can be reduced by folic acid and B-group vitamin administration in patients with PCOS on short-term metformin treatment. After 12 weeks, a 26.5% increase in Hcy levels was seen with metformin therapy, and 21.17 and 8.33% decreases in Hcy levels were seen in the B-group vitamin and the folic acid plus metformin groups respectively. These findings suggest that

Table III. Homocysteine, vitamin B_{12} and folate levels before and after treatment in the three groups (mean ± SEM)

Variable	Group	Before treatment	After treatment	Change from month 0 to 3 (95% CI)	*P*
Homocysteine (μmol/l)	1	9.56 ± 0.81	11.97 ± 1.01	+2.41 (+1.24, +3.59)	< 0.001
	2	11.43 ± 0.62	8.66 ± 0.49	−2.77 (−1.31, −4.23)	< 0.001
	3	11.96 ± 0.81	10.71 ± 0.70	−1.26 (−0.16, −2.35)	0.04
Folic acid (ng/ml)	1	8.32 ± 0.58	8.27 ± 1.10	−0.48 (−1.63, +1.53)	NS
	2	6.86 ± 0.71	7.28 ± 0.85	+0.42 (−1.23, +2.08)	NS
	3	8.03 ± 0.73	13.30 ± 1.93	+5.27 (+2.66, +7.89)	0.02
Vitamin B_{12} (pg/ml)	1	281.83 ± 24.51	226.08 ± 35.42	−55.75 (−10.35, +38.84)	NS
	2	236.27 ± 42.39	486.55 ± 66.26	+250.28 (+130.50, +370.06)	0.004
	3	219.05 ± 23.32	234.30 ± 26.91	+15.24 (−24.26, +54.54)	NS

Group 1 = metformin; Group 2 = metformin plus vitamin B_{12}; Group 3 = metformin plus folic acid.
CI = confidence interval; NS = not significant.

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Table IV. Glucose and lipid metabolism parameters before and after treatment (mean ± SEM)

Variable	Group	Before treatment	After treatment	Change from month 0 to 3 (95% CI)	*P*
Waist:hip ratio	1	0.81 ± 0.02	0.80 ± 0.02	−0.01 (−0.04, +0.02)	NS
	2	0.79 ± 0.02	0.77 ± 0.02	−0.02 (−0.05, +0.01)	NS
	3	0.80 ± 0.04	0.80 ± 0.02	0.00 (−0.03, +0.02)	NS
Weight (kg)	1	65.62 ± 3.71	64.73 ± 4.10	−0.89 (−3.10, +2.21)	NS
	2	65.81 ± 3.32	63.89 ± 2.91	−1.92 (−5.30, +1.46)	NS
	3	76.77 ± 4.15	77.44 ± 3.89	−0.68 (−4.86, +6.22)	NS
HOMA	1	3.07 ± 0.72	2.79 ± 0.39	−0.28 (−1.65, +1.08)	NS
	2	2.33 ± 0.30	2.52 ± 0.39	0.19 (−0.48, +0.86)	NS
	3	2.75 ± 0.39	3.16 ± 0.37	0.41 (−0.21, +1.02)	NS
Total-C (mg/dl)	1	178.07 ± 8.07	170.79 ± 5.57	+7.29 (−4.72, +19.29)	NS
	2	159.94 ± 5.62	162.50 ± 6.48	−5.56 (−14.34, +3.23)	NS
	3	174.13 ± 11.10	179.67 ± 11.19	−5.53 (−18.48, +7.42)	NS
HDL-C (mg/dl)	1	57.14 ± 4.94	57.86 ± 4.94	+0.71 (−1.63, +3.06)	NS
	2	51.94 ± 3.78	53.39 ± 3.28	+1.44 (−7.04, +9.93)	NS
	3	48.07 ± 3.40	48.88 ± 4.64	+0.82 (−7.54, +9.17)	NS
LDL-C (mg/dl)	1	104.29 ± 7.26	96.07 ± 5.66	−8.21 (−20.83, +4.40)	NS
	2	88.18 ± 4.15	91.88 ± 5.27	+3.70 (−3.77, +11.17)	NS
	3	98.27 ± 9.00	105.97 ± 7.61	+7.70 (−0.08, +15.47)	NS
VLDL-C (mg/dl)	1	18.86 ± 2.35	18.04 ± 3.31	−0.81 (−4.01, +2.38)	NS
	2	17.08 ± 2.34	16.83 ± 2.27	−0.24 (−3.29, +2.80)	NS
	3	27.69 ± 8.43	23.22 ± 5.18	−4.37 (−12.21, +3.47)	NS
TG (mg/dl)	1	94.43 ± 11.71	89.86 ± 16.52	−4.57 (−20.78, +11.64)	NS
	2	85.17 ± 11.62	84.61 ± 11.52	−0.56 (−15.74, +14.63)	NS
	3	138.53 ± 42.12	116.67 ± 25.94	−21.87 (−60.83, +17.09)	NS
Apo A_1 (mg/dl)	1	137.17 ± 8.47	153.33 ± 9.91	+16.17 (−1.82, +34.15)	NS
	2	147.50 ± 9.21	121.25 ± 10.79	−26.25 (−12.15, +2.72)	0.04
	3	124.71 ± 7.59	124.29 ± 12.84	−0.43 (−33.32, +32.70)	NS
Apo B (mg/dl)	1	85.17 ± 8.48	79.50 ± 10.36	−5.67 (−20.62, +9.29)	NS
	2	72.00 ± 7.54	67.29 ± 7.48	−4.71 (−12.15, +2.72)	NS
	3	76.27 ± 8.63	76.86 ± 9.74	+0.57 (−12.71, +13.85)	NS
Lp (a) (mg/dl)	1	45.80 ± 22.87	16.20 ± 3.54	−29.6 (−91.30, +32.10)	NS
	2	29.56 ± 9.68	32.22 ± 10.15	+2.67 (−1.51, +6.84)	NS
	3	43.57 ± 17.09	49.43 ± 15.68	+5.86 (−6.28, +17.99)	NS

Group 1 = metformin; Group 2 = metformin plus vitamin B_{12}; Group 3 = metformin plus folic acid.
CI = confidence interval; HOMA = insulin sensitivity calculated by homeostasis model assessment; Total-C = total cholesterol; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; VLDL-C = very low-density lipoprotein cholesterol; TG = triglyceride; Apo A_1 = apolipoprotein A_1; Apo B = apolipoprotein B; Lp (a) = lipoprotein (a); NS = not significant.

B-group vitamins and folic acid administration, especially B-group vitamins, can counteract the Hcy-increasing effects of metformin therapy.

Hcy is an essential amino acid required for the growth of cells and tissues in the human body. The only source of Hcy in the human organism comes from the methionine in dietary proteins, which are mainly of animal origin. Hcy, in turn, uses two pathways for biotransformation: trans-sulphuration and remethylation. Vitamin B_6 takes part in the process of trans-sulphuration, whereas folic acid, together with vitamin B_{12}, participates in the most important and more often used pathway for Hcy metabolism: remethylation. A reduction in dietary intake of B-group vitamins leads to an increase in levels of Hcy in plasma. Any drug, such as methotrexate, nitrous oxide or azaribine, that reacts with folic acid, vitamin B_{12} or B_6 respectively can cause hyperhomocysteinemia (de la Calle *et al.*, 2003). It has been previously demonstrated that metformin treatment decreases vitamin B_{12} levels (Wulffele *et al.*, 2003; Pongchaidecha *et al.*, 2004), probably due to malabsorption (Tomkin *et al.*, 1971; Caspary *et al.*, 1977). Hence, during metformin treatment, Hcy levels may increase (Carlsen *et al.*, 1997; Hoogeveen *et al.*, 1997; Aarsand and Carlsen, 1998). Therefore, it appears that both vitamin B_{12} and B_6 have important roles in Hcy regulation, and supplements with these vitamins may lower Hcy levels.

Abnormal fasting Hcy levels tend to reflect an impaired remethylation pathway, whereas an abnormal response to a methionine load points to a disturbed trans-sulphuration pathway. In our study, fasting Hcy levels increased significantly with metformin treatment. Although not statistically significant, we observed a 19.78% decrease in vitamin B_{12} levels in patients treated with metformin, while decreases in folic acid levels were minimal (0.6%). Therefore, it seems likely that a rise in Hcy levels may be related to a decrease in vitamin B_{12} levels. On the other hand, in the current study, Hcy levels decreased significantly with both metformin plus B-group vitamins and with metformin plus folic acid. Although there was no significant difference between the two groups with respect to the amount of Hcy reduction observed, decreases in Hcy levels were greater in patients treated with metformin plus B-group vitamins than they were in patients treated with metformin plus folic acid (2.77 ± 0.69 versus 1.26 ± 0.52 μmol/l). One explanation for this is that vitamin B_6 and vitamin B_{12} are two factors that act separately in Hcy metabolism and probably potentiate each other. We used a combination of vitamins B_1, B_6 and B_{12} because in Turkey there is no oral preparation that includes only vitamin B_{12}.

The strongest evidence to date that an elevated plasma Hcy concentration may be a risk factor for vascular disease

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comes from the study of individuals with homocystinuria. If left untreated, 25% of these individuals suffer a vascular event by the age of 16 years and 50% by the age of 29 years (Mudd *et al.*, 1985). Treatment of homocystinuria is directed at lowering Hcy concentrations by administering vitamin B_6, folate, betaine (a methyl donor), and vitamin B_{12}. This treatment has been shown to have a marked effect on preventing the occurrence of vascular events. Evidence from long-term follow-up of 158 patients with homocystinuria, with 2822 patient-years of treatment, has demonstrated that Hcy lowering, even to concentrations several times higher than the normal reference range, significantly reduces the incidence of vascular events (Yap *et al.*, 2001). Lowering Hcy levels in PCOS is important when one considers the elevated levels of Hcy and possible increasing effects of metformin on Hcy levels in patients with PCOS. Treatment of PCOS is symptomatic, but lifestyle measures, such as diet and exercise, could play an important role (Powell *et al.*, 1987). Although the exact mechanism is not known, regular exercise significantly lowers plasma Hcy in young, overweight or obese women with PCOS (Randeva *et al.*, 2002). Observational epidemiological studies, both case-controlled and cohort, demonstrate that patients with elevated Hcy levels have low to moderately increased risks of CVD. Folic acid (Jacobsen, 1998) and B-group vitamins (Woodside *et al.*, 1998) effectively lower Hcy concentration in plasma. However, at present, because of the lack of data from large-scale randomized trials, it remains unclear whether lowering Hcy levels will reduce the risk of CVD (Splaver *et al.*, 2004).

Because there is strong evidence for the use of metformin in the regulation of cycle and ovulation induction among patients with PCOS who desire fertility (Stadtmauer *et al.*, 2002), it should be remembered that this medication may interfere with vitamin B_{12} and folic acid pathways and lead to an increase in plasma Hcy levels (Desouza *et al.*, 2002). The association between increased levels of Hcy and neural tube defects and other congenital defects, spontaneous miscarriages, intrauterine growth retardation, pre-eclampsia, and intrauterine fetal death have been previously described (de la Calle *et al.*, 2003). Many authors suggest not only administration of folic acid but also vitamin B_{12} administration for prevention of neural tube defects (Czeizel and Dudas, 1992; Mills *et al.*, 1995; Nelen *et al.*, 1997; Brouwer, 2000; Nelen *et al.*, 2000), because vitamin B_{12} fosters cell intake of folic acid, so that even though concentrations of folic acid are normal, a deficiency of vitamin B_{12} also could increase the risk of neural tube defects (Smithells *et al.*, 1983; Czeizel and Dudal, 1992; Eskes, 1998; Leeda *et al.*, 1998; Brouwer, 2000). In our study, both B-group vitamins and folic acid decreased Hcy levels. B-Group vitamins are likely more effective than folic acid for lowering Hcy levels. Both vitamin B_{12} and folic acid should be offered to patients, especially those using metformin, who desire to become pregnant.

Although the sample size in the current study was small, these findings suggest that daily administration of folic acid or B-group vitamins may be effective in reducing elevated Hcy levels in patients with PCOS undergoing short-term metformin therapy.

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