

CLINICAL STUDY

Insulin resistance and endothelial function are improved after folate and vitamin B12 therapy in patients with metabolic syndrome: relationship between homocysteine levels and hyperinsulinemia

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Abstract

Objective: The purpose of this study was (a) to study whether a folate and vitamin B12 treatment, aimed at decreasing homocysteine levels, might ameliorate insulin resistance and endothelial dysfunction in patients with metabolic syndrome according to the National Cholesterol Education Program–Adult Treatment Panel-III criteria and (b) to evaluate whether, under these metabolic conditions, there is a relationship between hyperhomocysteinemia and insulin resistance.

Design and methods: A double-blind, parallel, identical placebo–drug, randomized study was performed for 2 months in 50 patients. Patients were randomly allocated to two groups. In group 1, patients were treated with diet plus placebo for 2 months. In group 2, patients were treated with diet plus placebo for 1 month, followed by diet plus folic acid (5 mg/day) plus vitamin B12 (500 µg/day) for another month.

Results: In group 2, folate treatment significantly decreased homocysteine levels by 27.8% (12.2 ± 1.2 vs 8.8 ± 0.7 µmol/l; $P < 0.01$). A significant decrement was observed for insulin levels (19.9 ± 1.7 vs 14.8 ± 1.6 µU/ml; $P < 0.01$) accompanied by a 27% reduction in the homeostasis model assessment levels. A positive relationship was found between the decrement of homocysteine and insulin levels ($r = 0.60$; $P < 0.002$). In parallel, endothelial dysfunction significantly improved in the treated group, since post-ischemic maximal hyperemic vasodilation increased by 29.8% and cGMP by 13.6% while asymmetrical dimethylarginine levels decreased by 21.7%. On the contrary, in group 1 patients, treated with placebo, no changes were shown in any of the variables.

Conclusions: Folate and vitamin B12 treatment improved insulin resistance and endothelial dysfunction, along with decreasing homocysteine levels, in patients with metabolic syndrome, suggesting that folic acid has several beneficial effects on cardiovascular disease risk factors.

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Introduction

It has been previously demonstrated that the administration of folic acid in patients with hyperhomocysteinemia leads to lower homocysteine levels by increasing the rate of recycling of homocysteine to methionine (1). *In vitro* studies have shown that homocysteine can directly damage endothelial cells (2), impairing the release or increasing the inactivation of nitric oxide (NO) (3) and, in turn, decreasing endothelial-dependent vasodilation (4). Conversely, it has been shown that folic acid treatment improves flow-mediated dilation in subjects with hyperhomocysteinemia (5, 6). Another

effect of homocysteine lies in its capacity to increase the production of asymmetrical dimethylarginine (ADMA), which emerged as an endogenously produced inhibitor of L-arginine, able to cause endothelial cell dysfunction with increased oxidative stress (7).

It is already known that the dysfunctional and uncoupled endothelial cell induced by homocysteine and ADMA becomes a net producer of damaging superoxide (O_2^-) instead of generating protective NO, a naturally occurring chain-breaking antioxidant (8). Oxygen reacts with the endothelial NO synthase (eNOS) enzyme in which tetrahydrobiopterin (BH4) cofactor is coupled to NAD(P)H to convert L-arginine to NO

and L-citrulline. When this cofactor is oxidized and does not function properly the entire eNOS reaction is uncoupled and the endothelium becomes a net producer of superoxide (O_2^-) radicals through the uncoupled NAD(P)H oxidase reaction (9). In addition to being a necessary cofactor, BH4 also serves as a naturally occurring antioxidant capable of scavenging reactive oxygen species such as O_2^- and peroxynitrite. A direct effect of folic acid in keeping BH4 functionally intact to maintain a coupled eNOS reaction has been demonstrated (10). In addition, folic acid was able to prevent NO synthase dysfunction induced by nitrate tolerance in the arterial circulation of healthy subjects (11) and pretreatment with oral folic acid prevented the endothelial dysfunction induced by acute hyperlipidemia following an oral fat load (12).

Controversial data on the association between insulin resistance and plasma homocysteine levels have been reported. In two large epidemiological studies, hyperhomocysteinemia was modestly but significantly associated with insulin levels and insulin resistance (13, 14). In addition, several case-control studies (15–20) have shown that insulin levels are significantly related to homocysteine levels in various groups of patients, such as hypertensive patients with type 2 diabetes, obese patients, women with polycystic ovary syndrome and pregnant women with pre-eclampsia. In contrast, Abbasi *et al.* (21) were unable to confirm such an association in a group of healthy subjects, and two other reports have indicated that an improvement in insulin sensitivity was not accompanied by an amelioration of homocysteine levels (22, 23). Whereas a few studies in animals have suggested that insulin affects the activity of the key enzymes of homocysteine metabolism (24), little is known about the relationship between homocysteine and insulin sensitivity in humans.

The possibility that folic acid might also act through an amelioration of insulin sensitivity in patients with metabolic syndrome might help to understand the beneficial effect of this drug in reducing cardiovascular risk factors in this class of patients.

Therefore, the aim of the present study was (a) to study whether a folate and vitamin B12 treatment, aimed at decreasing homocysteine levels, might ameliorate insulin resistance and endothelial dysfunction in patients with metabolic syndrome according to the National Cholesterol Education Program (NCEP)–Adult Treatment Panel (ATP)-III criteria (25) and (b) to evaluate whether, under these metabolic conditions, a decrease of homocysteine levels correlates with an amelioration of insulin sensitivity.

Materials and methods

Subjects

All subjects gave informed consent for their participation in the study and the local ethics committee

approved the protocol. We enrolled 50 patients with the following selection criteria: (a) presence of metabolic syndrome identified as fulfilling the NCEP–ATP-III criteria (25) and (b) the presence of hyperinsulinemia (Table 1). The anti-hypertensive therapy consisted of β -blockers, Ca antagonists and angiotensin-converting enzyme inhibitors, which were withdrawn 4 days before each test.

Study design

A double-blind, parallel, identical placebo–drug, randomized study was performed for 2 months. In particular, patients with metabolic syndrome were randomly allocated to two groups. In group 1, patients were treated with diet plus placebo for 2 months. In group 2, patients were treated for 1 month with diet plus placebo and then for 1 month with diet plus oral folic acid (folic acid, 5 mg/day (Schwarz Pharma, Milan, Italy)) plus vitamin B12 (500 μ g/day (Roche, Basel, Switzerland)) administration – subsequently referred as folate treatment – since recently published data have shown that folate and vitamin B12 are more effective in decreasing homocysteine levels than folate alone (26). Every 14 days, patients were reviewed by a dietician to maintain a constant body weight throughout the study and to equalize diet treatment between the two study groups.

At the end of each month of therapy, all subjects were admitted to the Metabolic Unit (part of Diabetology, Endocrinology, Metabolic Disease Unit) in the morning after an overnight fast, and samples were withdrawn after at least 30 min of resting in the supine position. After this period, basal forearm blood flow and systolic and diastolic blood pressure were measured. Maximal post-ischemic forearm blood flow was evaluated using venous occlusion plethysmography techniques, according to Capaldo *et al.* (27). To measure endothelial independent vasodilation, forearm blood flow was evaluated immediately before and 3 min

Table 1 Anthropometric and metabolic variables after 1 month of placebo therapy in 50 patients with metabolic syndrome (means \pm S.E.M.).

| | |
|--|-----------------|
| Gender: males–females | 41–9 |
| Age (years) | 67.3 \pm 1.2 |
| Weight (kg) | 79.5 \pm 1.5 |
| Body mass index (BMI) (kg/m ²) | 28.7 \pm 0.6 |
| Waist circumference (cm) | 125.3 \pm 1.2 |
| Systolic blood pressure (mmHg) | 164.5 \pm 2.0 |
| Diastolic blood pressure (mmHg) | 90.7 \pm 1.4 |
| Fasting glucose (mmol/l) | 6.3 \pm 0.1 |
| Fasting insulin (μ U/ml) | 19.6 \pm 1.0 |
| HbA1c (%) | 5.8 \pm 0.1 |
| Fasting triglycerides (mmol/l) | 3.59 \pm 0.14 |
| Fasting cholesterol (mmol/l) | 5.77 \pm 0.24 |
| Fasting HDL cholesterol (mmol/l) | 1.25 \pm 0.06 |
| Fasting creatinine (μ mol/l) | 75.2 \pm 2.25 |

HDL, high-density lipoprotein.

after the administration of isosorbide dinitrate (0.5 mg) (Nitrosorbide, Lusofamarco, Milan, Italy). The coefficient of variation (C.V.) of the test was 4.3%.

To evaluate fasting insulin resistance, the homeostasis model assessment (HOMA) index was used. The following formula was applied (glucose (mmol/l) × insulin (μU/ml)/22.5) (28).

Assays

Plasma glucose was measured with a glucose oxidase-based analyzer (Beckman Glucose Analyzer; Beckman, Fullerton, CA, USA). Serum insulin levels (intra-assay C.V. 3.0%, interassay C.V. 5.0%) were assayed with a microparticle enzyme immunoassay (MEIA) (IMX; Abbott Laboratories, Abbott Park, IL, USA). Serum triglyceride, cholesterol, high-density lipoprotein cholesterol and creatinine levels were measured using automated enzymatic spectrophotometric techniques adapted to Cobas Fara II (Roche). cGMP was assayed with an RIA kit (Amersham International plc, Amersham, Bucks, UK). Homocysteine was measured with an MEIA.

ADMA and symmetrical dimethylarginine (SDMA) were extracted from plasma samples using cation-exchange Strata SCX 100 mg columns (Phenomenex, Chemtek Analytica, Bologna, Italy) and assayed by high performance liquid chromatography as previously described (29).

Folate and vitamin B12 levels in serum were measured by RIA (DPC dual count solid phase kits; Diagnostic Products Corporation, Los Angeles, CA, USA).

Statistical analysis

All data are given as means ± S.E.M. Comparisons within groups were performed by Student's *t*-test for paired data. Comparisons among groups were performed by

ANOVA followed by the Scheffe *F* test when appropriate. Pearson and Spearman correlation coefficients were also used, where appropriate. A two-tailed probability level <0.05 was considered statistically significant. Multiple regression analysis was performed with changes in insulin levels after folate or placebo therapy as the dependent variable and all other parameters as independent variables. In addition, a similar analysis was performed using changes in maximal post-ischemic forearm blood flow after folate or placebo therapy as the dependent variable and all other parameters as independent variables.

Results

In Tables 2 and 3 are reported all the metabolic and endothelial variables under evaluation, before and after folate or placebo treatment. In group 2, folate treatment decreased homocysteine levels by 27.8% (12.2 ± 1.2 to 8.8 ± 0.7 μmol/l; *P* < 0.01) and ADMA levels by 21.7% (0.92 ± 0.08 to 0.72 ± 0.05 μmol/ml; *P* < 0.001). Interestingly, a similar percentage decrement was observed in insulin levels (25.6%, from 19.9 ± 1.7 to 14.8 ± 1.6 μU/ml; *P* < 0.01) and in HOMA levels, the index of fasting insulin resistance (27%, from 5.48 ± 0.43 to 4.00 ± 0.43; *P* < 0.01). A significant improvement in endothelial function was achieved, as demonstrated by an increment in post-ischemic maximal hyperemic vasodilation by 29.8% (from 18.1 ± 1.3 to 23.5 ± 1.6 ml/100 ml forearm per min; *P* < 0.001), while endothelial-independent vasodilation remained unchanged. A possible involvement of the NO pathway was suggested by the fact that increased cGMP levels, the second messenger of NO, were shown (from 2.16 ± 0.18 to 2.96 ± 0.27 pmol/ml; *P* < 0.01). In parallel, a slight but significant decrease in systolic blood pressure was also demonstrated. On the contrary, no changes were demonstrated in all these parameters in group 1.

Table 2 Changes in anthropometric and metabolic variables in patients with metabolic syndrome after 2 months of placebo therapy (group 1; 25 patients) or after 1 month of placebo followed by 1 month of folate therapy (group 2; 25 patients). Values are mean ± S.E.M.

| | Group 1 (21 male/4 female) | | | Group 2 (20 male/5 female) | | |
|------------------------------------|----------------------------|----------------------|--------------|----------------------------|---------------------|---------------|
| | Placebo 1st month | Placebo 2nd month | Difference | Placebo 1st month | Folate 2nd month | Difference |
| Body weight (kg) | 79.1 ± 2.4 | 78.8 ± 2.3 | -0.32 ± 0.43 | 79.8 ± 1.8 | 78.7 ± 2.0 | -1.10 ± 1.23 |
| BMI (kg/m ²) | 28.1 ± 0.7 | 28.0 ± 0.6 | -0.1 ± 0.1 | 29.4 ± 0.8 | 28.7 ± 0.7 | -0.7 ± 0.3 |
| Systolic blood pressure (mmHg) | 165 ± 2 | 165 ± 3 | 0 ± 2 | 164 ± 4 | 154 ± 2† | -10 ± 3* |
| Diastolic blood pressure (mmHg) | 92 ± 2 | 93 ± 2 | 1 ± 1 | 89 ± 2 | 85 ± 2 | -4 ± 2 |
| Fasting glucose (mmol/l) | 6.24 ± 0.08 | 6.16 ± 0.08 | -0.08 ± 0.10 | 6.21 ± 0.18 | 5.98 ± 0.16 | -0.23 ± 0.12 |
| Fasting insulin (μU/ml) | 19.3 ± 1.3 | 19.0 ± 1.13 | -0.4 ± 1.0 | 19.9 ± 1.7 | 14.8 ± 1.6‡ | -5.72 ± 1.10* |
| HOMA | 5.38 ± 0.38 | 5.21 ± 0.33 | -0.17 ± 0.32 | 5.48 ± 0.43 | 4.00 ± 0.43‡ | -1.48 ± 0.30* |
| Fasting triglycerides (mmol/l) | 3.75 ± 0.18 | 3.56 ± 0.22 | -0.18 ± 0.21 | 3.43 ± 0.23 | 2.82 ± 0.51† | -0.61 ± 0.18 |

**P* < 0.01 vs treatment with placebo; †*P* < 0.05 vs placebo (1st month); ‡*P* < 0.01 vs placebo (1st month).

Table 3 Changes in endothelial variables in patients with metabolic syndrome after 2 months of placebo therapy (group 1; 25 patients) or after 1 month of placebo followed by 1 month of folate therapy (group 2; 25 patients). Values are mean \pm S.E.M.

| | Group 1 (21 male/4 female) | | | Group 2 (20 male/5 female) | | |
|---|----------------------------|----------------------|------------------|----------------------------|---------------------|-------------------|
| | Placebo 1st month | Placebo 2nd month | Difference | Placebo 1st month | Folate 2nd month | Difference |
| Folate (ng/ml) | 13.2 \pm 0.7 | 13.4 \pm 0.8 | 0.3 \pm 0.7 | 12.4 \pm 1.0 | 29.2 \pm 2.6¶ | 16.9 \pm 2.5† |
| Vitamin B12 (pg/ml) | 293 \pm 17 | 294 \pm 15 | 0 \pm 14 | 302 \pm 17 | 489 \pm 24¶ | 186 \pm 18† |
| Homocysteine (μ mol/l) | 11.0 \pm 0.7 | 10.8 \pm 0.3 | -0.2 \pm 0.6 | 12.2 \pm 1.2 | 8.8 \pm 0.7¶ | -3.4 \pm 0.8* |
| cGMP (pmol/ml) | 2.26 \pm 0.14 | 2.33 \pm 0.14 | 0.10 \pm 0.20 | 2.16 \pm 0.18 | 2.96 \pm 0.27‡ | 0.80 \pm 0.20* |
| ADMA (μ mol/ml) | 0.95 \pm 0.08 | 0.93 \pm 0.07 | -0.02 \pm 0.02 | 0.92 \pm 0.08 | 0.72 \pm 0.05¶ | -0.21 \pm 0.05* |
| SDMA (μ mol/ml) | 0.61 \pm 0.05 | 0.62 \pm 1.13 | 0.01 \pm 0.08 | 0.63 \pm 0.06 | 0.61 \pm 0.05 | -0.02 \pm 0.04 |
| Basal forearm blood flow ^a | 2.68 \pm 0.16 | 2.50 \pm 0.14 | -0.18 \pm 0.15 | 2.51 \pm 0.16 | 2.65 \pm 0.16 | 0.14 \pm 0.17 |
| Maximum post-ischemic forearm blood flow ^a | 19.2 \pm 1.3 | 16.7 \pm 1.0 | -2.5 \pm 1.2 | 18.1 \pm 1.3 | 23.5 \pm 1.6‡ | 5.4 \pm 1.5† |
| Endothelial-independent vasodilation in forearm blood flow ^a | 5.26 \pm 0.38 | 5.31 \pm 0.34 | -0.05 \pm 1.8 | 5.34 \pm 0.30 | 5.39 \pm 0.30 | -0.05 \pm 0.20 |

^a ml/100 ml forearm per min.

* $P < 0.01$ vs placebo; † $P < 0.001$ vs placebo; ‡ $P < 0.05$ vs placebo (1st month); ¶ $P < 0.001$ vs placebo (1st month).

In an attempt to better understand the effects of folate treatment on the metabolic and endothelial variables, we analyzed the differences in their values at the end of the placebo or the folate treatment compared with those at the start of both treatments in terms of net increment or decrement, and the data are reported in Tables 2 and 3 under the columns labeled 'Difference'. As expected, in group 2, along with a significant decrement in homocysteine (-0.2 ± 0.6 vs -3.4 ± 0.8 μ mol/l; $P < 0.01$) and ADMA levels (-0.02 ± 0.02 vs -0.21 ± 0.05 μ mol/ml; $P < 0.001$), there was a significant decrement in insulin (-0.4 ± 1.0 vs -5.72 ± 1.10 μ U/ml; $P < 0.01$), HOMA (-0.17 ± 0.32 vs -1.48 ± 0.30 ; $P < 0.01$) and systolic blood pressure (0 ± 2 vs -10 ± 3 mmHg; $P < 0.01$) (Table 2). On the contrary, significant increments in cGMP levels (0.10 ± 0.20 vs 0.80 ± 0.20 pmol/ml; $P < 0.01$) and post-ischemic maximal hyperemic vasodilation (-2.5 ± 1.2 vs 5.4 ± 1.56 ml/100 ml forearm per min; $P < 0.001$) were observed (Table 3).

Pooling all the data together, homocysteine levels were positively correlated with insulin ($r = 0.31$, $P < 0.002$), HOMA ($r = 0.29$, $P < 0.005$) and triglyceride ($r = 0.24$, $P < 0.02$) levels. Finally, ADMA levels were negatively correlated with post-ischemic maximal hyperemic vasodilation ($r = -0.27$, $P < 0.01$). In addition, cGMP levels were positively correlated with post-ischemic maximal hyperemic vasodilation ($r = 0.27$, $P < 0.01$) and negatively correlated with insulin ($r = -0.33$, $P < 0.0001$) and HOMA ($r = -0.32$, $P < 0.0002$) levels. In group 2, the analysis of the difference between placebo vs folate treatment showed that the decrement in homocysteine levels was significantly correlated with the decrement in insulin levels ($r = 0.60$, $P < 0.002$; Fig. 1).

In a multiple regression analysis, changes in homocysteine levels independently correlated with changes

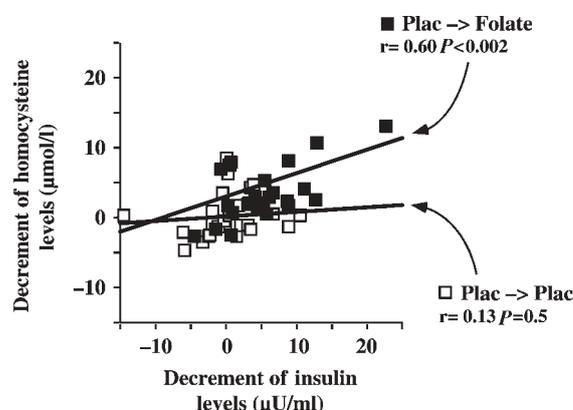


Figure 1 Relationship between the decrement in homocysteine and the decrement in insulin levels during folate (■) and placebo (□) treatment. A positive relationship was found between the two parameters during folate treatment while no relationship was found during placebo administration.

in insulin levels ($P < 0.002$; data not shown). In addition, changes in maximal post-ischemic forearm blood flow were independently correlated with changes in cGMP ($P < 0.005$; data not shown) and ADMA levels ($P < 0.01$; data not shown).

Discussion

The present study has shown, for the first time, that a prolonged folate treatment decreased not only homocysteine levels but also reduced insulin levels, improving insulin resistance in patients with metabolic syndrome. Furthermore, it improved endothelial function as demonstrated by an increment in cGMP levels and maximal post-ischemic forearm blood flow and decreased ADMA levels. Our work seems to strengthen

the evidence that folic acid treatment has various beneficial effects in reducing the risk factors of cardiovascular disease, as recently reviewed (30).

The most interesting result of the study relates to the relationship between the decrement of homocysteine levels by folate treatment and the reduction of insulin levels, with an amelioration of insulin sensitivity in severe insulin-resistant patients, adding new data on the controversial relationship between insulin and homocysteine.

Also, in our study, the correlation coefficient between insulin and homocysteine levels was $r = 0.60$, with results similar to those reported in three studies in both rats and humans (18). We were not able to add any new insights on the possibility that insulin modulates homocysteine levels, as suggested in rats (24), or whether it is homocysteine that is able to modulate insulin levels. An action of homocysteine on the insulin secretory pathway was demonstrated by Najib & Sanchez-Margalet (31), who found that homocysteine thiolactone, the active form of homocysteine, inhibited the insulin-stimulated tyrosine phosphorylation of insulin receptor β -subunit and its substrates insulin receptor substrate-1 and p60-70 in rat hepatoma cells (31). In addition, they showed that homocysteine thiolactone decreased the p85 regulatory subunit of phosphatidylinositol 3-kinase activity, inducing a reduction in insulin-stimulated glycogen synthesis (31). In that study, the effects of 100 nM insulin were completely blocked by 50 μ M homocysteine thiolactone, suggesting that, in this *in vitro* study, 1 μ M homocysteine thiolactone inhibited $\sim 300 \mu$ U/ml insulin, partly explaining the result of our *in vivo* study in which a 3.4 μ mol/l decrement of homocysteine levels was associated with a decrease in circulating insulin levels of 5 μ U/ml, but this important issue deserves further investigation.

However, it must be underlined that the effect of folate treatment in reducing insulin resistance is very important since our group previously demonstrated that insulin resistance and decreased NO release after an oral glucose tolerance test, as an index of endothelial dysfunction, are independent predictors of restenosis after stent implantation in patients with cardiovascular disease (32). Thus, a treatment able to ameliorate both insulin resistance and endothelial dysfunction might be very helpful in the treatment of cardiovascular disease in patients with metabolic syndrome.

A potential bias of our study might be due to the confounding effect of prolonged diet therapy on insulin sensitivity in the patients treated with folate treatment. However, in placebo-treated patients, no significant effect of prolonged diet treatment was demonstrated. In addition, body weight did not change during the 6 months before and throughout the study, and reviews by a dietitian were performed every 2 weeks to maintain a constant diet during all study periods,

both placebo and folate treatment. In addition, the significant increment in serum folate and vitamin B12 levels suggests a good compliance of patients to treatment. All these points made us trust the true effect of folate treatment on insulin sensitivity.

Another possible confounding factor could be related to genetic determinants of homocysteine metabolism causing different effects of folate therapy on insulin resistance. At present only two studies have evaluated the effects of the C677T polymorphism of 5,10-methylenetetrahydrofolate reductase in subjects with insulin resistance and no significant correlations were found between this polymorphism and homocysteine levels and/or common features of insulin resistance (33, 34).

The data from the present study underline the effects of folate treatment in improving endothelial function as suggested by the improvement of maximal post-ischemic vasodilation, associated with a reduction in systolic blood pressure. These confirm previous results on the effects of folate on vascular endothelial function, as recently reviewed (30). In addition, we were able to find a 20% decrease in ADMA levels after a month of folate treatment, a figure in agreement with previous data reporting a 30% decrease in ADMA after 3 months of therapy with metformin and peroxisome proliferator-activated receptor γ (PPAR γ) agonists (35, 36). The importance of folate treatment in reducing ADMA levels in hyperhomocysteinemic patients has also been reported by Holven *et al.* (37). On the contrary, different results have been reported by Sydow *et al.* (38), who found that treatment with 10 mg folate and 200 μ g vitamin B2 daily for 8 weeks, although it significantly decreased homocysteine levels, was unable to influence both endothelial vasodilation and ADMA levels. One possible explanation is related to the different degree of atherosclerosis present in the three studies (18). In fact, in the present study and in that of Holven *et al.* (37), patients were affected by metabolic syndrome and hyperhomocysteinemia in the absence of peripheral vascular disease while in Sydow *et al.* (38) patients had severe peripheral arterial occlusive disease. All in all, these results strongly underline the importance of starting folate therapy as early as possible in these patients, when endothelial dysfunction could be still reversible.

Previous studies have demonstrated that homocysteine could regulate ADMA metabolism by inhibiting dimethyl arginine dimethylaminohydrolase (DDAH) activity. Stühlinger *et al.* (39) suggested that homocysteine, binding DDAH, could form mixed disulfides capable of blocking ADMA binding (39). This mechanism might explain why hyperhomocysteinemia could lead to an increase in ADMA levels by reducing NO elaboration from endothelial cells. Another possible mechanism explaining the effects of homocysteine in increasing ADMA levels has been proposed by Boger *et al.* (40) studying human endothelial cells in

the presence of a high concentration of homocysteine. They postulated that an increase in methionine availability could result in increased ADMA formation (40). In accordance with Stühlinger *et al.* (39), the present study, providing evidence that folate treatment decreases homocysteine levels and in turn induces a significant decrement in ADMA but not SDMA levels, seems to support the hypothesis that ADMA levels are regulated by alterations in DDAH activity.

An effect of folate treatment mediated by an NO pathway seems to be strengthened by the increased circulating cGMP levels found in the present study in patients with metabolic syndrome after 1 month of folate treatment. It is interesting to note that circulating cGMP levels before folate therapy were similar to those found in type 2 diabetic patients while, after folate therapy, cGMP was significantly increased to levels found in normal subjects (41). To our knowledge, these data with regard to the effects of folate on cGMP levels have never been reported in humans but are in accordance with previous results in rats, demonstrating that ischemia-induced angiogenesis was rescued by oral folate via an NO-dependent mechanism (42). In particular, the authors found increased tissue nitric oxide and, in turn, cGMP concentrations which correlated with increased serum folate levels, supporting our data of a folate-dependent increase in cGMP levels, a new mechanism of action of folate treatment.

On the other hand, folate treatment could improve endothelium-dependent vasodilation by other metabolic pathways. First, folic acid reduces homocysteine levels by increasing the rate of recycling of homocysteine to methionine (1), preventing homocysteine-induced oxidative stress and consequently endothelial injury. Secondly, folic acid decreases the formation of NO₂⁻ anions and peroxynitrite induced by high homocysteine concentrations restoring the NO response to bradykinin and L-arginine. In fact, Stroes *et al.* (43) demonstrated that in the presence of decreased availability of tetrahydrobiopterin, an essential cofactor of eNOS, there was a shift in the balance between the precursors of NO and oxygen free radicals by the enzyme. Thirdly, folic acid could improve endothelial function *per se* as recently shown by Doshi *et al.* (44), who found that acute oral administration of 5 mg folate significantly increased flow-mediated dilation and that this effect was independent of changes in homocysteine levels. However, data in the present study suggest that folate acts mainly through the different pathways previously proposed, while the direct effects of folate probably have only a secondary role.

In conclusion, in patients with metabolic syndrome, folate treatment improved insulin resistance and endothelial dysfunction, along with decreasing homocysteine and ADMA levels, suggesting that folic acid may have several beneficial effects on cardiovascular disease risk factors.

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