ORIGINAL ARTICLE

No effect of folic acid on markers of endothelial dysfunction or inflammation in patients with type 2 diabetes mellitus and mild hyperhomocysteinaemia

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ABSTRACT

Background: Mild hyperhomocysteinaemia is a cardiovascular risk factor in patients with type 2 diabetes mellitus. Homocysteine may exert its detrimental effects through induction of endothelial dysfunction and/or chronic inflammation. In this study, we examined the effects of homocysteine-lowering therapy with folic acid on biochemical markers of endothelial dysfunction and low-grade inflammation in patients with type 2 diabetes mellitus and mild hyperhomocysteinaemia (≥ 14 μmol/l).

Methods: In a randomised, double-blind, controlled trial, patients were treated with folic acid 5 mg or placebo for six months. At 0 and 6 months, albuminuria, von Willebrand factor, soluble cellular adhesion molecules, C-reactive protein, interleukin-6 and tumour necrosis factor-α were determined.

Results: Forty-one patients completed the study (folic acid 23, placebo 18). Baseline hyperhomocysteinaemia (median 17 μmol/l, range 14 to 30 μmol/l) was reduced by 29% in the folic-acid-treated group, and remained unchanged in patients receiving placebo. On average, folic acid treatment did not significantly affect any of the endothelial (e.g. von Willebrand factor: difference folic acid minus placebo +1%, confidence interval -3 to +16%) or inflammation (e.g. C-reactive protein: difference folic acid minus placebo +13%, confidence interval -42 to +52%) markers studied. Multiple regression analyses without and with adjustment for baseline differences in cardiovascular disease and ethnicity confirmed these results. An apparent beneficial effect of folic acid on albuminuria in crude analysis was attenuated by multiple adjustment (difference folic acid minus placebo -35%, confidence interval -178 to +32%, p=0.08, adjusted 0.26).

Conclusion: The data indicate that, in this group of patients with type 2 diabetes mellitus and mild hyperhomocysteinaemia, lowering homocysteine with folic acid for six months does not improve biochemical markers of endothelial dysfunction or low-grade inflammation.

INTRODUCTION

Mild hyperhomocysteinaemia is a recently discovered, independent risk factor for cardiovascular disease.[1] Patients with type 2 diabetes mellitus may be particularly vulnerable to the atherothrombotic effects of hyperhomocysteinaemia when compared with nondiabetic subjects.[2,3]
The pathophysiological basis for the association between hyperhomocysteinaemia and cardiovascular disease is unclear. Studies in animals and humans suggest that hyperhomocysteinaemia increases oxidative stress inducing, amongst other things, endothelial dysfunction.[4,5] Another

proposed mechanism is promotion of the chronic inflammatory component of atherosclerosis by homocysteine.[6] The increased susceptibility to hyperhomocysteinaemia of patients with type 2 diabetes mellitus is unexplained, but may relate to the association of both high homocysteine and diabetes with endothelial dysfunction and chronic, low-grade inflammation which particularly occur in individuals with type 2 diabetes whose urinary albumin excretion is increased.[7,8] In addition, recent animal data suggest that homocysteine induces superoxide formation particularly in the vascular endothelium of diabetic rabbits.[9]

Folic acid lowers the serum homocysteine concentration.[10] However, whether this translates into clinical benefit is still unknown. Randomised controlled trials among nondiabetic individuals have shown that homocysteine-lowering treatment can improve intermediate clinical endpoints, i.e. decrease of restenosis after angioplasty,[11] and decreased incidence of abnormal exercise electrocardiography tests.[12] In addition, homocysteine lowering may improve endothelium-dependent vasodilation,[13] although the latter is not a consistent finding.[14] Studies addressing the effects of folic acid on biochemical markers of endothelial function or chronic inflammation as surrogate markers of cardiovascular disease are scarce, and show conflicting results.[15,16] In patients with diabetes mellitus the effects of folic acid treatment have only sporadically been studied, despite their reported increased vulnerability to hyperhomocysteinaemia.

In view of these considerations, we investigated the effects of treatment with folic acid on biochemical markers of endothelial dysfunction and chronic inflammation in patients with type 2 diabetes mellitus, mild hyperhomocysteinaemia and a high-normal to clearly elevated urinary albumin excretion.

METHODS

Patients

Patients aged 30 to 85 years, with a ≥1 year history of type 2 diabetes mellitus, a fasting homocysteine concentration of ≥14 µmol/l and a urinary albumin-to-creatinine ratio of at least 1 mg/mmol in early morning urine at initial screening were considered for inclusion. Exclusion criteria were unstable glycaemic control (defined as more than 1.5% absolute change in HbA1c during the previous year), plasma vitamin B_{12} or folate concentrations outside the reference range (see below), serum creatinine >130 µmol/l, blood pressure >160 mmHg and/or >95 mmHg, congestive heart failure, major invalidating disease (e.g. severe pulmonary disease, cancer), severe hyperlipidaemia (total cholesterol >7.5 mmol/l or triglycerides >5 mmol/l), and pregnancy.

Study protocol

Patients were randomised between treatment with 5 mg folic acid or similar placebo tablets. The random allocation sequence was generated by computer and implemented by numbered containers. Both participants and physicians were unaware of group assignment.

At baseline and at three and six months of follow-up, subjects came to the hospital in the morning, after a ten-hour fasting period. We measured blood pressure in the sitting position after ten minutes of rest with a standard clinical sphygmomanometer, blood glucose, HbA1c, serum creatinine, serum total cholesterol, HDL cholesterol and triglycerides. At baseline and after six months, plasma levels of total homocysteine, vitamin B_{12}, folate, von Willebrand factor, soluble E-selectin, soluble vascular cell adhesion molecule-1, soluble intercellular adhesion molecule-1, C-reactive protein, interleukin-6 and tumour necrosis factor-α were measured. In addition, urinary albumin and creatinine excretion was measured in triplicate first-voided morning urine samples.[17] During the study period, medication used for treatment of hypertension or hyperlipidaemia was not changed, unless the treating physician decided that blood pressure or lipids were in an unacceptable range, in which case patients were excluded.

Laboratory analyses

Plasma and serum aliquots were quickly separated and frozen at -30 °C and -70 °C for batched analysis. HbA1c was measured using an automated high performance liquid chromatography analyser (Diamat BioRad Laboratories, NY, USA, reference range 5.2 to 6.7%). Serum and urinary creatinine was assayed with a modified Jaffé method. Total serum cholesterol was measured using a fully enzymatic (CHOD-PAP) kinetic method. HDL cholesterol was measured with the same method after precipitation of very-low-density and low-density lipoprotein with phosphotungstic acid and magnesium ions. Serum triglycerides were determined using an enzymatic method. Total plasma homocysteine was measured with high performance liquid chromatography, using a previously described method.[18] For better separation, chromatographic conditions were changed into gradient elution from 0 to 20% acetonitril in 0.1 M KH2PO4 (pH 1.75). Serum vitamin B_{12} and folate were measured with a competitive protein-binding assay (Dualcount Solid Phase Boil assay, DPC, Los Angeles, CA, USA; reference values 150 to 700 pmol/l and 6.8 to 39 nmol/l, respectively). Urinary albumin was measured using a laser-nephelometric method on a Behring Nephelometer (Behringwerke, Germany). Von Willebrand factor antigen levels were measured, in duplicate, by an enzyme-linked immunosorbent assay, using polyclonal antibodies from Dako (Glostrup, Denmark) and expressed as percentage of von Willebrand factor

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detected in pooled citrate plasma of healthy controls. Soluble cell adhesion molecules were assayed in plasma by ELISAs obtained from Diaclone (Besançon, France; intracellular and intercellular assay coefficients of variation are 4.0 and 10.8% for soluble E-selectin; 4.4 and 7.6% for s-vascular cell adhesion molecule-1 (sVCAM-1); and 4.0 and 8.6% for s-intercellular adhesion molecule-1 (sICAM-1), respectively). Reference values obtained in 40 healthy volunteers were 54.3 to 126.9 ng/ml for sE-selectin, 250 to 799 ng/ml for sICAM-1 and 761 to 1510 ng/ml for sVCAM-1. C-reactive protein levels were measured in plasma by a latex-enhanced immuno-turbidimetric method (Roche Tinaquant) performed on a Roche/Hitachi modular P800. Interleukin-6 was measured in serum by a sandwich enzyme immunoassay (Quantikine High Sensitivity, R&D Systems, Oxon, United Kingdom; intra- and inter-assay coefficients of variation <11.1% and <16.5%, respectively). Tumour necrosis factor-α was measured in serum by a sandwich enzyme immunoassay (Quantikine High Sensitivity, R&D Systems, Oxon, United Kingdom; intra- and inter-assay coefficients of variation <8.8% and <16.7%, respectively).

Statistical analyses

Data are expressed as mean (standard deviation), or as median (interquartile range). We used regression models to determine differences in outcome variables between the treatment groups, and to perform multiple adjustments for potential confounders and baseline values. We constructed general scores for markers of endothelial dysfunction and of inflammatory activity to reduce the influences of biological variability of each measure. For each individual, the values of each marker were expressed as a Z score, i.e (value in the individual minus the mean value in the study population) divided by the standard deviation, a value that thus ranged from approximately -2.5 to + 2.5. The general score of endothelial marker proteins was then calculated as the mean of the Z scores for von Willebrand factor, soluble E-selectin, soluble vascular cell adhesion molecule-1, and soluble intercellular adhesion molecule-1. Likewise, the general score for inflammatory markers was calculated as the mean of the Z scores for C-reactive protein, interleukin-6, and tumour necrosis factor-α. The study was designed to have a 90% power (1-β) to detect a difference between the treatment groups the size of 1 SD of each studied variable.

RESULTS

Fifty-one patients started this study. Seven patients declined further cooperation within three months, and one was lost to follow-up. Two patients were excluded because of serious protocol violations (intake of folic acid before start of study), leaving 41 patients for final analyses (*figure 1*). Because of
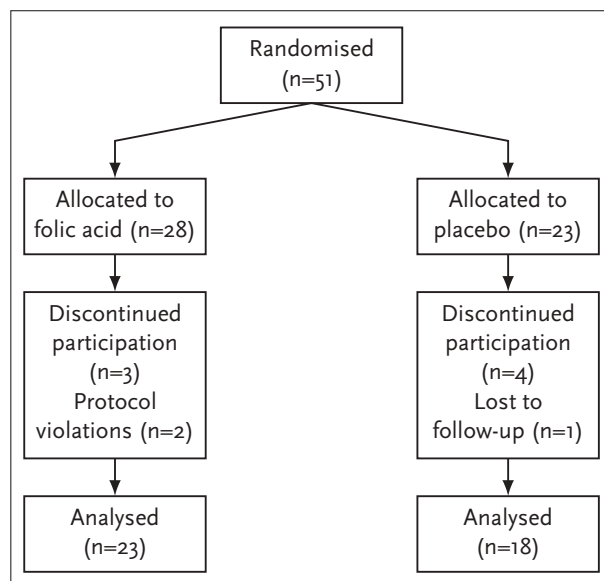
Figure 1
Flow diagram of the study

this relatively high dropout rate, and in view of the reasons for dropout, we performed on-treatment analysis of the data. Most demographic, clinical and laboratory characteristics at baseline of patients who completed the study were comparable in both treatment groups (*table 1*). Somewhat less patients in the placebo group were non-Caucasian or had a history of cardiovascular disease.

Six months of folic acid therapy resulted in a significant decrease in homocysteine concentration of 29%. Analysis of the crude data showed that, on average, markers of endothelial dysfunction and inflammation did not change differently between the placebo and folic acid group (*table 2, figure 2*). Multiple linear regression analyses using baseline-adjusted values at t=6 months as the dependent variables revealed no significant effect of folic acid compared with placebo on protein markers of endothelial dysfunction and inflammation (*table 3*). There was a trend towards improvement of albuminuria after six months of treatment with folic acid compared with placebo (*table 3*, p=0.08). However, adjustment for baseline differences in ethnicity and cardiovascular disease attenuated this result.

Blood pressure, lipid levels, vitamin B_{12} and HbA1c remained stable during the trial and were not affected by treatment with folic acid (data not shown).

DISCUSSION

In this randomised placebo-controlled trial of patients with type 2 diabetes mellitus, mild hyperhomocysteinaemia and high-normal to elevated urinary albumin excretion, folic

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Table 1

Baseline characteristics of the patients

	FOLIC ACID (N = 23)	PLACEBO (N = 18)
Clinical characteristics		
Age (years)	63.7 (8.6)	66.1 (8.5)
Sex (% male)	61	56
Ethnicity (% non-Caucasian)	35	17
Duration of diabetes (years)	11 (4- 19)	13 (7- 19)
Oral hypoglycaemic agents (%)	48	39
Body mass index (kg/m2)	29.3 (3.9)	28.8 (3.4)
Systolic blood pressure (mmHg)	139.4 (22.7)	140.8 (15.7)
Diastolic blood pressure (mmHg)	76.1 (12.0)	75.6 (10.3)
Smoking (%)	39	50
Cardiovascular disease (%)	44	67
Use of angiotensin-converting enzyme-inhibitors or Angiotensin II-antagonists (%)	78	74
Biochemical variables		
HbA1c (%)	7.6 (1.3)	7.3 (1.2)
Serum creatinine (μmol/l)	106 (21)	104 (21)
Serum cholesterol (mmol/l)	5.1 (1.0)	4.8 (1.1)
Serum triglyceride (mmol/l)	1.76 (1.17-1.96)	1.24 (0.85-2.10)
Serum HDL cholesterol (mmol/l)	1.1 (0.6-1.9)	1.1 (0.7-3.2)
Homocysteine (μmol/l)	17 (15-21)	18 (15-20)
Serum vitamin B_{12} (pmol/l)	236.6 (63.2)	263.5 (85.1)
Serum folate (nmol/l)	13.4 (4.6)	11.7 (3.3)
Endothelial dysfunction		
Albumin excretion (mg/mmol creatinine)	4.8 (1.2-15.4)	2.2 (1.2-32.3)
Microalbuminuria (%)	74	55
Macroalbuminuria (%)	13	22
Von Willebrand factor (%)	173 (126-231)	192 (129-258)
Soluble E-Selectin (ng/ml)	118 (85-158)	139 (97-169)
S-vascular cell adhesion molecule-1 (ng/ml)	1347 (1070-1640)	1399 (1078-1576)
S-intercellular adhesion molecule-1 (ng/ml)	668 (598-865)	797 (513-1046)
Inflammation		
C-reactive protein (mg/l)	2.8 (1.2-5.3)	3.5 (1.2-15.7)
Interleukin-6 (pg/ml)	4.5 (2.9-5.5)	5.4 (3.2-10.2)
Tumour necrosis factor-α (pg/ml)	2.5 (2.2-3.5)	2.4 (1.9-3.5)

Baseline clinical and laboratory characteristics of the patients who completed the study. Continuous data are indicated as mean (standard deviation), or in case of data with skewed distributions, as median (interquartile range). Nominal data are presented as percentages.
Cardiovascular diseases = myocardial infarction, coronary artery bypass grafting, percutaneous transluminal (coronary) angioplasty, amputation or stroke.
Microalbuminuria = albumin/creatinine ratio 1 to 30 mg/mmol, macroalbuminuria = albumin/creatinine ratio >30 mg/mmol.

acid treatment for six months did not decrease the levels of putative markers of endothelial dysfunction and inflammation. Point estimates of effect size were around zero for practically all marker proteins. Albumin excretion showed some improvement in the folic-acid-treated group, but this effect was insignificant after adjustment for confounders.

The rationale for using these marker proteins is that they reflect endothelial damage (urinary albumin/creatinine ratio), regulation of platelet adhesion and aggregation (von Willebrand Factor), leucocyte adhesion (soluble E-selectin, soluble vascular cell adhesion molecule-1 and soluble intercellular adhesion molecule-1), and chronic, low-grade inflammation (C-reactive protein, interleukin-6 and tumour necrosis factor-α). Elevated plasma levels of these markers are associated with an increased risk in cardiovascular disease.[19-21] Furthermore, von Willebrand factor, soluble vascular cell adhesion molecule-1, soluble intercellular

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Table 2

Percentage change of biochemical markers after six months of treatment

	FOLIC ACID	PLACEBO	DIFFERENCE (CI) FOLIC ACID MINUS PLACEBO
Δ homocysteine (%)	-29 (-56 to 0)	-10 (-27 to +35)	-19 (-43 to -12)
Δ albumin excretion (%)	-13 (-100 to +464)	22 (-64 to +104)	-35 (- 178 to +32)
Δ von Willebrand Factor (%)	3 (-10 to +32)	2 (-36 to +43)	+1 (-3 to +16)
Δ sE-selectin (%)	4 (-25 to +64)	5 (-43 to 123)	-1 (-27 to +7)
Δ sVCAM-1 (%)	-1 (-33 to +25)	-1 (-41 to +79)	0 (-15 to +10)
Δ sICAM-1 (%)	0 (-45 to +64)	2 (-29 to +71)	-2 (-11 to +15)
Δ C-reactive protein (%)	24 (-59 to +176)	11 (-99 to +313)	+13 (-42 to +52)
Δ interleukin-6 (%)	8 (-55 to +159)	1 (-94 to +107)	+7 (-58 to +42)
Δ TNF-α (%)	0 (-38 to 124)	-5 (-42 to +81)	+5 (-14 to +18)

Percentage change of homocysteine and marker levels after six months of treatment with folic acid /placebo, indicated as median (range), difference between both groups with 95% confidence intervals (CI).
vWF = von Willebrand factor, sE-selectin = soluble E-selectin, sVCAM-1 = soluble vascular cell adhesion molecule-1, sICAM-1 = soluble intercellular adhesion molecule-1, CRP = C-reactive protein, IL-6 = interleukin-6, TNF-α = tumour necrosis factor-α.

adhesion molecule-1 and tumour necrosis factor-α were previously shown to correlate with homocysteine levels in patients with diabetes mellitus.[22,23] Because the sensitivity and the specificity of individual marker proteins is limited,[24] we used two panels of markers for endothelial dysfunction and low-grade inflammation.

This is, to the best of our knowledge, the first study investigating two panels of markers for endothelial dysfunction and low-grade inflammation in patients with type 2 diabetes mellitus and mild hyperhomocysteinaemia. Only one study investigated a comparable patient group, but investigated other biochemical markers, i.e. of haemostatic and fibrinolytic abnormalities (plasminogen activator inhibitor type 1, protein C activity, antithrombin III activity, fibrinogen, thrombomodulin). Treatment with folic acid and pyridoxine did not decrease their levels.[25]
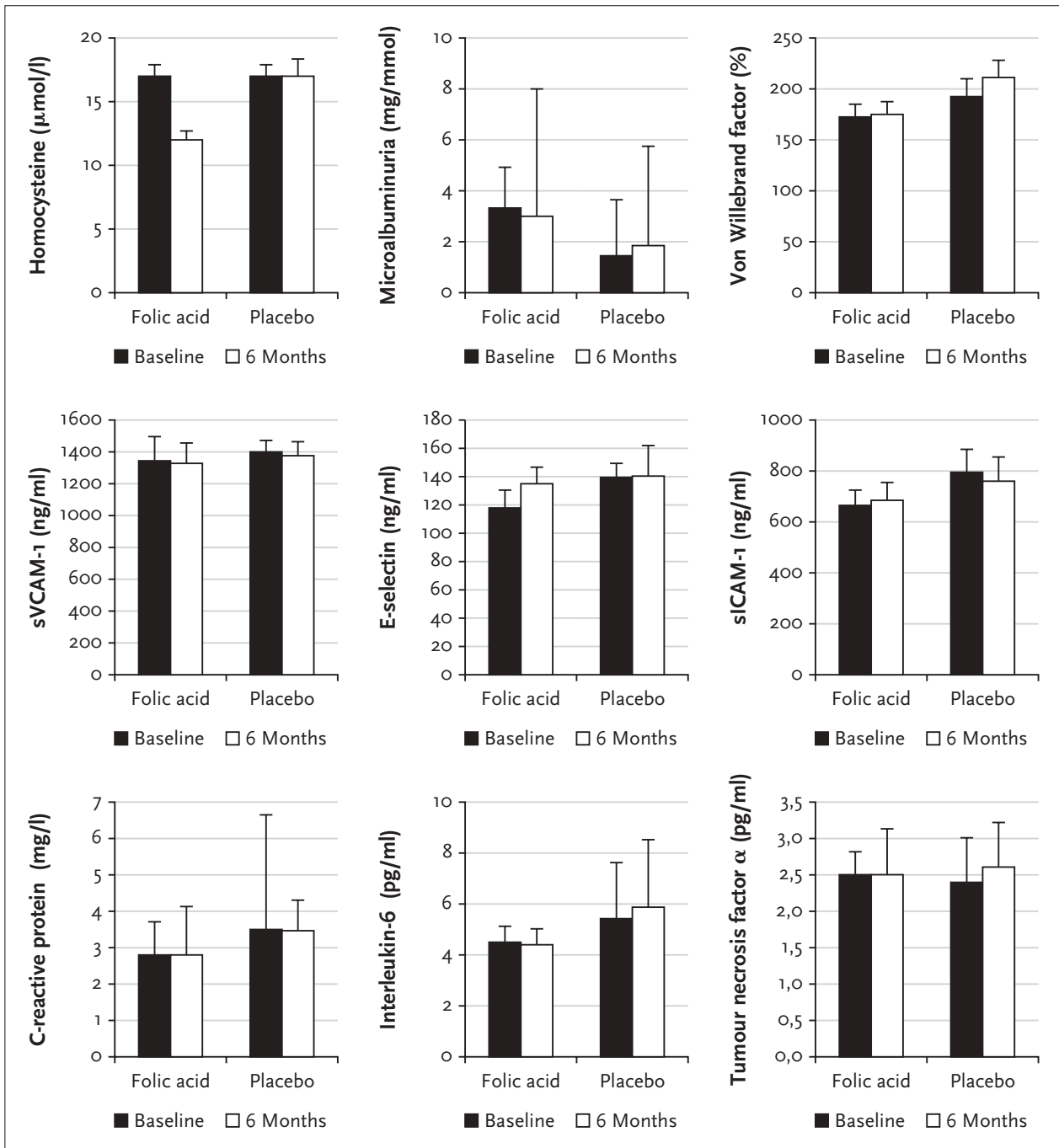
Other studies have been performed in nondiabetic populations. In general, the effects on the separate markers were comparable with our results. Administration of folic acid, in some studies combined with pyridoxine and/or vitamin B₁₂, consistently decreased homocysteine levels. Von Willebrand factor was most frequently used as biochemical marker of endothelial dysfunction. Five out of six studies showed no effect of treatment with folic acid on vWF concentration.[16,26-29] Only in one study using high-dose folic acid (10 mg) were vWF levels decreased.[30] Adhesion molecules were only sporadically determined in B-vitamin intervention trials. Folic acid did not decrease their levels,[16,31] except in one study, in which folic acid was combined with polyunsaturated fatty acids and oleic acid, reducing levels of VCAM-1.[32] One study addressed the effect of

homocysteine-lowering treatment on albuminuria.[16] In this nondiabetic population albuminuria decreased by 20% after two years, which roughly approximates the crude effect size estimate in our study. However, urinary albumin-to-creatinine ratio in this other study was mostly within the 'normal' reference range, and subjects were treated with both folic acid and pyridoxine. Further study is clearly necessary to resolve this issue.
Very few studies, all in nondiabetic subjects, investigated the effect on inflammation markers. Levels of C-reactive protein and interleukin-6 did not decrease,[15,16,29,31] TNF-α levels were not investigated.

Most studies demonstrating a beneficial effect of folic acid on endothelial dysfunction involved endothelium-dependent vasodilation rather than biochemical endothelial tests.[13,33-36] However, the results are not unequivocal as a number of studies showed no effect of homocysteine-lowering therapy on flow-mediated dilatation.[14,37,38] In patients with type 2 diabetes, local intra-arterial administration of 5-methyltetrahydrofolate (the active form of folic acid) acutely restored endothelium-dependent vasodilation.[39] It is not clear, however, that this effect is related to lowering of homocysteine, as folate may improve endothelial vasomotor function by homocysteine-independent mechanisms.[40]

The main limitation of this trial is the relatively limited number of subjects. Nevertheless, point estimates of effect size were around zero for practically all marker proteins, making major effects on endothelial function and inflammation unlikely. Furthermore, we used combined scores consisting of sets of markers to increase statistical power.

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Figure 2
Vertical bars with median values of the folic acid and placebo group at baseline and after six months for homocysteine, albumin/creatinine ratio, von Willebrand Factor and C-reactive protein: effects of folic acid on endothelial function and inflammation.

Nevertheless, larger studies are clearly needed to assess whether small or moderate effects of folate treatment on endothelial marker proteins exist. Another limitation is the relatively large number of patients who did not finish the trial after randomisation, making on-treatment analysis necessary. On the other hand, pathophysiological studies such as this one are less vulnerable in this respect than trials with clinical endpoints. Also, the dropout number was about equal in both patient groups and therefore probably did not significantly influence the results.

In summary, our study shows that beneficial effects of homocysteine-lowering therapy, should they turn out to exist for clinical endpoints, are not likely to be predictable by measuring biochemical markers of endothelial damage or low-grade inflammation. Patients with type 2 diabetes, in

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Table 3

Effect of folic acid vs placebo treatment on outcome variables: multiple regression analysis

DEPENDENT VARIABLE	MODEL I			MODEL II		
	B	CI	P	B	CI	P
Homocysteine	0.77	0.68 to 0.87	<0.005	0.77	0.66 to 0.88	<0.005
Albuminuria	0.53	0.27 to 1.09	0.084	0.65	0.30 to 1.38	0.26
VWF	1.06	0.96 to 1.16	0.25	1.06	0.94 to 1.17	0.32
sE-selectin	0.91	0.78 to 1.07	0.27	0.90	0.76 to 1.08	0.25
sVCAM-1	0.98	0.88 to 1.11	0.78	0.98	0.86 to 1.11	0.69
sICAM-1	0.99	0.89 to 1.16	0.83	0.99	0.86 to 1.14	0.90
Panel A	0.62	0.21 to 1.79	0.37	0.53	0.17 to 1.73	0.29
CRP	1.12	0.65 to 1.90	0.69	0.95	0.58 to 1.92	0.86
IL-6	0.82	0.53 to 1.28	0.37	0.82	0.51 to 1.30	0.38
TNF-α	0.97	0.87 to 1.22	0.70	0.98	0.84 to 1.16	0.82
Panel B	0.90	0.26 to 3.16	0.87	0.61	0.15 to 2.36	0.46

Multiple regression analyses with B coefficients for treatment assignment (placebo = 0; folic acid = 1) as independent variable and different outcome variable levels as dependent variables.
Model 1: baseline-value-adjusted only. Model 2: after additional adjustment for baseline differences in ethnicity and cardiovascular disease.
Panel A = set of markers for endothelial dysfunction, calculated as sum of the z-scores. Panel B = set of markers for low-grade inflammation, calculated as sum of the z-scores.
vWF = von Willebrand factor, sE-selectin = soluble E-selectin, sVCAM-1 = soluble vascular cell adhesion molecule-1, sICAM-1 = soluble intercellular adhesion molecule-1, CRP = C-reactive protein, IL-6 = interleukine-6, TNF-α = tumour necrosis factor-α.
Dependent variables were ln-transformed because of ln-normal distribution. After regression analysis antilogarithmic transformation of the B coefficients and confidence interval values was performed. Because of the back transformation, B values of 0-1 indicate a negative effect of folic acid (and are thus comparable with negative B values in normal data). Confidence intervals containing 1 should be interpreted as statistically not significant.

spite of being more susceptible to mild hyperhomocysteinaemia, appear to be no exception to this rule. Endothelium-dependent vasodilation is likely to be more sensitive in detecting improved endothelial function due to folate treatment.

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