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No effect of folic acid supplementation in the course of 1 year on haemostasis markers and C-reactive protein in older adults

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Summary

Elevated homocysteine levels are associated with an increased cardiovascular disease (CVD) risk, but the underlying mechanism is still unclear. High homocysteine might affect the endothelium, and consequently lead to impaired haemostasis. In a randomized placebo controlled trial among 276 older adults with plasma total homocysteine concentrations above 13 mM at screening, we investigated the effect of homocysteine lowering by folic acid supplementation (0.8 mg/day) for 1 year on markers of endothelial function (von Willebrand factor), coagulation (tissue factor, factor VIIa, fragments 1+2), and fibrinolysis (fibrin degradation products, tissue-type plasminogen activator), and inflammation (C-reactive protein). Despite a 24% reduction in plasma homocysteine concentration and four-fold increase in serum folate concentration in the folic acid group compared to the placebo group, there was no clear change in any of the haemostasis markers, nor CRP. Although homocysteine is associated with vascular disease risk in the general population, marked lowering of slightly elevated homocysteine concentrations by one-year folic acid supplementation does not influence haemostasis markers.

Keywords

Homocysteine, folic acid, haemostasis, homocysteine, intervention

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Introduction

Elevated homocysteine levels are associated with an increased cardiovascular disease (CVD) risk (1). However, homocysteine levels can be effectively lowered by supplementation with folic acid (3). Homozygosity for the 677C>T mutation in the gene encoding methylenetetrahydrofolate reductase (MTHFR), an enzyme involved in homocysteine remethylation, has been associated with a 16% increase in risk of coronary heart disease (CHD) (4). This finding supports the hypothesis that an impaired homocysteine or folate metabolism is causally related to CHD, although the mechanism is still unclear. Animal and *in vitro* studies have suggested that homocysteine might damage the endothelium, thereby inducing atherothrombosis by stimulation of procoagulant pathways and impairment of anti-coagulant and fibrinolytic pathways (5). However, observational and intervention studies in humans have shown mixed results (6–26).

In this study we investigated the effect of homocysteine reduction by folic acid supplementation for 1 year on various markers of haemostasis including von Willebrand Factor (VWF), tissue factor (TF), factor VIIa (FVIIa), prothrombin fragment 1+2 (F_{1+2}), fibrin degradation products (FbDP), tissue-type plasminogen activator activity (tPA-activity) in volunteers aged 50–70. The inflammation marker C-reactive protein (CRP) was evaluated because it has been proposed that elevated homocysteine levels contribute to low-grade inflammation (27), and changes in haemostasis might be related to changes in inflammatory condition (28).

Materials and methods

Subjects

This study is part of a larger placebo controlled double blind trial among 819 older adults volunteers examining the effect of folic acid supplementation for 3 years on the progression of carotid artery intima-media thickness. In this sub-trial we focused on the effect of 1-year folic acid supplementation on haemostasis markers in 276 subjects who were randomized between Septem-

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ber and December 2000. Subjects were recruited from local blood banks and municipal registries in Wageningen and surroundings. Subjects were eligible if they were between 50 and 70 years old, had no thyroid or renal disease, did not use drugs that affect homocysteine or progression of intima-media thickness (i.e. hormone replacement or lipid lowering therapy), and did not use B-vitamin supplements. Women were postmenopausal or in case of hysterectomy >55 years. At screening, plasma homocysteine values had to be between 13 and 26 µM, and serum vitamin B_{12} concentrations >200 pM. The study was approved by the medical ethics committee of the Wageningen University; written informed consent was obtained from all participants.

Design

During a screening visit blood was taken to determine homocysteine and serum vitamin B_{12} concentrations. After a 6-week placebo run-in period, subjects were randomized to oral treatment with folic acid (0.8 mg/d) or placebo in capsule-form (Swiss Caps Benelux, Roosendaal, The Netherlands). At baseline and after one year, blood was collected. Compliance was judged by pill-return counts and a calendar that registered missed pills, both of which were returned every twelve weeks. All subjects fulfilled our compliance criterion which was defined as >80% of capsules taken.

Blood collection and analysis

After the subject had been seated for 15 minutes, fasting blood samples were drawn into evacuated vials without moving the needle in the vein. Blood used for determination of serum vitamin B_{12}, folate and lipids was collected in serum separator vials, which were placed in the dark and stored at room temperature for 30–60 min before centrifugation. Blood used for determination of plasma homocysteine concentration was collected in EDTA containing vials, for determination of FVIIA, VWF, TF, F_{1+2} and CRP in citrate-containing vials, and for determination of tPA in Stabilyte vials. Where necessary, vials were directly put on ice until centrifugation. The plasma was separated from blood cells within 30 minutes by centrifugation at 2000 x g for 20 min at the appropriate temperature. Samples were quickly frozen using dry-ice and stored at –80°C until analysis. Pre- and post-intervention samples of a participant, except for folate and vitamin B_{12}, were analyzed in the same run to minimize variability. Folate and vitamin B_{12} concentrations were measured with a commercial chemiluminescent immunoassay analyzer (Immulite 2000, Diagnostic Products Company, Los Angeles, USA). The intra-assay coefficients of variation (CV) of these assays were all <10%. Creatinine concentrations and lipid profile were determined by Hitachi 747. Total plasma homocysteine concentrations were measured by HPLC with fluorimetric detection (intra- and interassay CV 2% and 7%, respectively) (29). Commercial kits were used to determine plasma concentrations of TF (Actichrome TF, American Diagnostica, Greenwich, USA), Factor VIIa (StaClot VIIa-rTF, Stago, Asnières, France), F_{1+2} (Enzygnost, Dade-Behring, Marburg, Germany), FbDP (Fibrinostika®FbDP, Organon Technika, Boxtel, The Netherlands), and to measure tPA-activity (Chromalize t-Pa, Biopool, Umeå, Sweden). Home-made ELISA with polyclonal antibodies was used for determination of VWF and high sensitive CRP concentration (Dako, Glastrup, Denmark). The intra- and interassay CVs were all <10%, except for FVIIa (intra-assay CV<15%), and for TF (inter-assay CV<15%). Genomic DNA was isolated from whole blood. The MTHFR 677C>T genotype was determined by PCR and restriction enzyme digestion with *Hin*FI, according to Frosst et al. (30). All laboratory staff was blinded for the treatment allocation.

Other measurements

A self-reported medical history was attained by questionnaire and reviewed by a research assistant. The questionnaire included questions on smoking, prevalent diseases, current drug use and family history of premature vascular disease (onset <60 years in first degree family). A participant was considered to have prevalent vascular disease if he had been diagnosed with angina pectoris, myocardial infarction, arrhythmia, stroke, or peripheral arterial disease or if he had undergone balloon angioplasty, coronary bypass surgery or aortic aneurysm surgery. Height and weight was measured to calculate body mass index. Blood pressure was measured using an automated meter (Dinamap® Compact Pro, General Electric) in supine position. The average of eight measurements was taken. Hypertension was defined as systolic blood pressure ≥160 mmHg, diastolic blood pressure ≥95 mmHg or use of antihypertensive medication. Hypercholesterolaemia was defined as total cholesterol <6.5 mM or high density lipoprotein cholesterol <0.9 mM.

Statistics

Descriptive data are shown as means ± SD, medians and interquartile range or frequencies. Differences at baseline between the folic acid and placebo group were tested by student *t* tests or Pearson's chi-square tests, for continuous and categorical variables respectively. Skewed continuous data were natural log transformed before testing. Pearson's correlation coefficients were calculated to examine the relation between folate and homocysteine concentrations on the one hand with markers of haemostasis and CRP on the other hand in the total population at baseline.

Baseline and 1-year values, and changes of homocysteine, folate, haemostasis markers and CRP are presented as medians and interquartile range. Per subject, treatment effect was calculated as the percentage change relative to baseline values. For all variables, the percentage change was not normally distributed and we therefore used a natural log transformation. Differences in (natural log transformed) baseline values and changes between the two intervention groups were tested with student *t* tests (a=0.05). Analysis of variance was used to investigate whether the MTHFR 677 C>T polymorphism influenced treatment effects. Data-analysis was performed with the Statistical Application Software for PC, version 6.12 (SAS institute Inc., Cary, North Carolina, USA).

Results

The trial comprised 139 participants in the placebo group and 137 participants in the folic acid group (Table 1). Values of conventional cardiovascular risk factors, and vitamin B_6 and B_{12} levels were in the normal range and not statistically different be-

Table 1: Baseline characteristics.[1]

	Placebo (n=139)	Folic Acid (n=137)
Age, y	60.2 ± 5.2	59.5 ± 5.8
BMI, kg/m^2	26.8 ± 3.9	26.8 ± 3.9
Vitamin B_{12}, pM[1]	300 (175 – 715)	286 (153 – 735)
Vitamin B_6, nM[1]	32 (24 – 44)	34 (24 – 44)
Total cholesterol, mM	5.6 ± 0.8	5.6 ± 1.0
HDL cholesterol, mM	1.2 ± 0.3	1.2 ± 0.3
LDL cholesterol, mM	3.8 ± 0.7	3.8 ± 0.9
Systolic blood pressure, mmHg	131 ± 14	130 ± 17
Diastolic blood pressure, mmHg	76 ± 8	77 ± 9
Creatinine, µM	92 ± 11	95 ± 13
Alcohol intake, g/d	16.0 ± 14.1	14.7 ± 13.9
Sex, % M/F	70/30	77/23
MTHFR genotype, % CC/CT/TT	34/57/10	37/42/19*
Hypercholesterolemia, %	25.2	31.4
Hypertension, %	21.7	26.7
Diabetes, %	2.2	3.7
Prevalent vascular disease (VD), %	8.6	11.7
Current use of VD related drugs, %	5.0	5.8
Current smoking, %	23.7	17.5

[1]Data are presented as mean ± SD or %, except for vitamin B_6 and B_{12} which are presented as median (interquartile range); * P<0.05 versus placebo.

tween groups. Prevalence of diabetes mellitus and CVD or use of CVD-related drugs did also not differ between the two groups. The MTHFR 677TT genotype was more prevalent and the CT genotype less prevalent in the folic acid group compared with the placebo group (P<0.05).

Table 2: Baseline and 1-year concentrations and percentage change of folate, homocysteine and haemostasis markers.[1]

	Placebo (n=139)	Folic Acid (n=137)	*P* value [2]
Folate			
Baseline, nM	13.3 (10.4; 15.5)	12.4 (10.2; 15.6)	
After 1 year, nM	11.4 (9.2; 14.5)	49.5 (42.3; 90.3)	
Change, %	–11 (-26; +5)	+308 (213; 640)	0.0001
Homocysteine			
Baseline, µM	12.5 (11.1; 14.6)	13.1 (11.8; 14.8)*	
After 1 year, µM	12.1 (10.6; 14.5)	9.6 (8.8; 10.5)	
Change, %	–2 (-10; +7)	–26 (-33; –18)	0.0001
Tissue Factor			
Baseline, pg/ml	138 (99; 209)	124 (83; 177)	
After 1 year, pg/ml	142 (105; 192)	145 (82; 184)	
Change, %	–3 (-20; +18)	+8 (-17; +29)	0.0872
Factor VIIa			
Baseline, mU/ml	69 (54; 86)	70 (53; 86)	
After 1 year, mU/ml	72 (56; 93)	72 (55; 95)	
Change, %	+6 (-14; +34)	0 (-12; +22)	0.7850
F_{1+2}			
Baseline, nM	0.69 (0.58; 0.85)	0.67 (0.58; 0.82)	
After 1 year, nM	0.82 (0.70; 1.01)	0.81 (0.65; 0.97)	
Change, %	+15 (-2; +36)	+13 (-4; +30)	0.5213
FbDP			
Baseline, ng/ml	199 (136; 269)	203 (119; 266)	
After 1 year, ng/ml	215 (145; 277)	189 (131; 282)	
Change, %	+3 (-18; +31)	+3 (-17; +25)	0.9812
tPA-activity			
Baseline, IU/ml	0.53 (0.33; 0.80)	0.50 (0.31; 0.75)	
After 1 year, IU/ml	0.61 (0.40; 0.84)	0.55 (0.34; 0.84)	
Change, %	+10 (-18; +57)	3 (-22; +51)	0.9781
VWF			
Baseline, %	94 (68; 138)	92 (70; 141)	
After 1 year, %	99 (71; 147)	95 (70; 129)	
Change, %	+1 (-19; +26)	–1 (-25; +20)	0.0989
CRP			
Baseline, mg/l	0.9 (0.5; 2.3)	1.2 (0.6; 2.2)	
After 1 year, mgl	1.0 (0.6; 1.8)	1.0 (0.6; 1.9)	
Change, %	+3 (-34; +62)	–8 (-34; +37)	0.4180

[1]Median (interquartile range); [2]Differences in changes between the two intervention groups were tested with student *t* tests; *P<0.05 versus placebo group.

At baseline, homocysteine and folate were not correlated with any of the haemostasis markers (data not shown). As shown in table 2, baseline concentrations of folate, vitamin B12, haemostasis markers and CRP were in the normal range and not different between both treatment groups. Baseline homocysteine concentration was significantly higher in the folic acid group (Table 2), which might be due to the higher prevalence of the TT genotype.

After 1-year supplementation, mean folate concentration was significantly increased by 319% (=36 nM) and mean homocysteine concentration significantly decreased by 24% (=3.1 mM) in the folic acid group, compared with the placebo group (Table 2). Folic acid supplementation did not affect haemostasis markers, nor CRP (Table 2).

Although subjects with the MTHFR 677TT polymorphism had significantly higher baseline homocysteine concentrations, lower folate concentrations (data not shown) and exhibited a greater homocysteine reduction, the polymorphism did not appear to influence the effect of folic acid supplementation on markers of haemostasis or CRP (Table 3).

Discussion

In this randomized placebo controlled trial among middle-aged volunteers, folic acid supplementation for 1 year did not have an effect on haemostasis markers or CRP, despite a considerable reduction in homocysteine (≈24%) and a large increase in serum folate (≈319%) concentrations. Although normal to marginally elevated homocysteine levels are associated with increased risk of vascular disease, it is unlikely that homocysteine acts by influencing haemostasis in the general population.

To our knowledge, this is the first large placebo-controlled intervention trial that examined the effect of homocysteine-lowering at various stages of the coagulation pathway, together with markers of endothelial function and inflammation performed in a representative sample of older adults without overt CVD. Although the subjects were recruited from the general population, they are believed to be at higher risk of CVD as they were selected based on moderately elevated plasma total homocysteine concentrations (≥13 µM). For most of the measured markers there is considerable evidence that they are positively associated with (31, 32) or even predict risk of CVD (10, 33–38).

Our hypothesis was that homocysteine impairs the endothelium such that coagulation is enhanced. Adverse effects on the endothelium should be reflected by an increase in VWF (39). Homocysteine has been shown to increase transcription and activity of TF from macrophages in the vessel wall (40–43), and therefore might stimulate the release of TF from the vessel wall. This would lead to an increased state of activation of the extrinsic coagulation cascade (increasing levels of FVIIa and F_{1+2}) and subsequently reactive fibrinolysis (increasing tPA-activity and FbDP concentrations). CRP was evaluated, because elevated homocysteine levels may contribute to low-grade inflammation (27), and inflammation may lead to coagulation activation (28). Although we expected that homocysteine lowering by folic acid supplementation would decrease the concentration or activity of VWF, haemostasis markers, and CRP, no effect of folic acid supplementation on any of the markers was observed. In addi-

Table 3: Percentage change for folate, homocysteine and haemostasis markers within MTHFR 677C>T genotypes.[1]

	Placebo[2]	Folic acid[3]	*P* value[4]
Folate			
CC	–9 (-28; +5)	+294 (+214; +606)	0.0001
CT	–11 (-24; +8)	+360 (+256; +729)	0.0001
TT	–18 (-31; –11)	+280 (+163; +659)	0.0001
Homocysteine			
CC	–1 (-8; +5)	–22 (-27; –16)	0.0001
CT	–4 (-10; +8)	–26 (-32; –16)	0.0001
TT	+1 (-7; +15)	–34 (-43; –26)	0.0001
Tissue Factor			
CC	0 (-20; +20)	+5 (-18; +29)	0.2632
CT	–4 (-20; +22)	+8 (-19; +37)	0.2759
TT	–9 (-25; +2)	+12 (-13; +22)	0.3686
Factor VIIa			
CC	+9 (-8; +34)	–4 (-14; +11)	0.0570
CT	+6 (-19; +36)	+1 (-12; +28)	0.6820
TT	+6 (-20; +34)	+11 (-5; +25)	0.4201
F_{1+2}			
CC	+15 (-11; +39)	+13 (+1; +27)	0.7364
CT	+14 (+2; +31)	+13 (0; +30)	0.9145
TT	+18 (-3; +36)	+18 (-1; +44)	0.3677
FbDP			
CC	+4 (-19; +37)	+1 (-19; +25)	0.9593
CT	+1 (-16; +30)	+4 (-14; +24)	0.6373
TT	+11 (-23; +31)	+9 (-16; +32)	0.7620
tPa-activity			
CC	+6 (-14; +38)	+1 (-21; +102)	0.5090
CT	+12 (-18; +57)	+4 (-22; +50)	0.9424
TT	+45 (-20; +95)	+15 (-32; +42)	0.1161
VWF			
CC	+3 (-18; +38)	+6 (-26; +31)	0.7511
CT	0 (-23; +20)	–7 (-26; +13)	0.0412
TT	+2 (-20; +61)	–3 (-15; +14)	0.4547
CRP			
CC	+10 (-25; +85)	–10 (-34; +7)	0.1886
CT	+3 (-39; +31)	–7 (-44; +43)	0.9484
TT	–5 (-35; +44)	+13 (-27; +80)	0.8018

[1]Median (interquartile range); [2]CC n=47, CT n=78, TT n=14; [3]CC n=50, CT n=57, TT n=26; [4]Differences in changes between the two intervention groups were tested with student *t* tests.

tion, homocysteine concentration was not correlated with any of the haemostasis markers at baseline.

Our results are not likely explained by poor quality of plasma samples used for determination of haemostasis markers since we followed a strict protocol for sampling and handling of the blood. Furthermore, the CVs of haemostasis markers were all below 15%. However, three possible explanations are conceivable for our results.

First, given the uncertainty regarding the mechanism underlying increased atherothrombotic risk in association with elevated homocysteine levels, it is possible that relevant haemostatic mechanisms are not being monitored by the assays chosen for analysis. However, two other placebo-controlled trials studying the effect of B-vitamin supplementation on haemostasis in healthy volunteers did not find any clear effect, although also other markers than ours were measured in these trials (21, 22, 26).

Second, results from a 6-month trial on in-stent restenosis by Lange et al. (47) suggest that positive effects of folic acid treatment may be greater in those with baseline homocysteine levels ≥15 μM. The average homocysteine concentrations of our participants might have been too low to have adversely affected haemostasis. At screening, we selected people with a homocysteine ≥13 mM. However at baseline, only 46% of the subjects still had homocysteine values ≥13 mM, probably due to regression to the mean. This might have lowered the power of our trial. In a non-placebo controlled trial, Undas et al. (48) supplemented 17 healthy subjects with fasting homocysteine levels >16 μM with a high dose of B-vitamins for 8 weeks and found a significant reduction of F_{1+2} and thrombin-antithrombin. However, we did not find any difference in effect on haemostasis or CRP when we stratified the analyses according to baseline homocysteine values (data not shown). In addition, we did not observe any difference in effect between MTHFR 677C>T genotypes, which is in line with the results from two other studies (21, 24).

In three studies, acute hyperhomocysteinemia induced by methionine loading significantly increased levels of VWF, F_{1+2}, D-dimer, tPA and PAI (7, 49, 50). However, it could be questioned whether these acute large effects on homocysteine could be compared with chronically elevated fasting homocysteine levels. Some studies in homocystinuria patients suggest that chronically high homocysteine levels are associated with increased haemostatic dysbalance (51).

Third, in our middle aged population, levels of haemostasis factors were relatively low compared to vascular patients (6, 32, 35, 45), and possibly could not be reduced any further. Even in the highest tertile of haemostasis markers or CRP, no effect of folic acid supplementation could be observed (data not shown).

Our results do not exclude possible effects in patients with compromised endothelial function. Several studies investigated the effect of high dose folic acid supplementation for a period of three months to one year on haemostasis in vascular patients. One of these studies showed a significant reduction in VWF (18). Two studies did not find an effect on the same markers as we measured in our study (19, 20), whereas two other studies observed significant effects on some other haemostasis markers (23, 24). However, it should be noted that most of these studies did not include a placebo group and sample sizes were generally small. Moreover, results of a placebo-controlled trial in 285 patients with previous transient ischaemic attack or stroke recently showed that homocysteine-lowering did not significantly reduce blood concentrations of biomarkers of inflammation, endothelial dysfunction, or hypercoagulability (52).

In conclusion, our findings indicate that it is unlikely that slightly elevated homocysteine levels increase the risk of a first CVD event through effects on haemostasis. More randomized trials are needed to evaluate whether homocysteine lowering would have a beneficial effect on haemostasis among subjects with documented clinical manifestation of CVD.

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