ORIGINAL RESEARCH

Effect of Combined Treatment With Folic Acid, Vitamin B_6, and Vitamin B_{12} on Plasma Biomarkers of Inflammation and Endothelial Dysfunction in Women

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***Background*—**The aim of this study was to determine whether reducing plasma homocysteine concentrations with long-term, combined treatment with folic acid, vitamin B_6, and vitamin B_{12} alters plasma biomarkers of inflammation and endothelial dysfunction in women at increased risk of cardiovascular disease.

***Methods and Results*—**We conducted a blood substudy of 300 treatment-adherent participants (150 in the active treatment group, 150 in the placebo group) in the WAFACS (Women's Antioxidant and Folic Acid Cardiovascular Study), a randomized, double-blind, placebo-controlled trial testing a daily combination of folic acid (2.5 mg), vitamin B_6 (50 mg), vitamin B_{12} (1 mg), or matching placebo, in cardiovascular disease prevention among women at increased risk of cardiovascular disease. Plasma concentration of 3 biomarkers of inflammation (C-reactive protein, interleukin-6, and fibrinogen) and a biomarker of endothelial dysfunction (intercellular adhesion molecule 1) were measured at baseline and at the end of treatment and follow-up. After 7.3 years of combined treatment with folic acid, vitamin B_6, and vitamin B_{12}, homocysteine concentrations were reduced by 18% in the active treatment group as compared with the placebo group ($P<0.001$). However, there was no difference between treatment groups in change in blood concentration from baseline to follow-up for C-reactive protein ($P=0.77$), interleukin-6 ($P=0.91$), intercellular adhesion molecule 1 ($P=0.38$), or fibrinogen ($P=0.68$).

***Conclusions*—**These findings indicate that long-term, combined treatment with folic acid, vitamin B_6, and vitamin B_{12} lowers homocysteine concentrations, but does not alter major biomarkers of vascular inflammation, consistent with the lack of clinical cardiovascular disease benefit in the trial.

***Clinical Trial Registration*—**URL: http://www.clinicaltrials.gov. Unique identifier: NCT00000541. **(*J Am Heart Assoc*. 2018;7: e008517. DOI: 10.1161/JAHA.117.008517.)**

Key Words: B vitamins • biomarkers • cardiovascular disease • endothelial dysfunction • inflammation • randomized trial

Mild or moderate hyperhomocysteinemia, typically defined as fasting blood homocysteine concentrations between 12 and 30 μmol/L,[1,2] has been associated with increased risks of cardiovascular disease (CVD) in observational studies.[3] However, evidence supporting a causal relationship is lacking. In vitro studies and studies in animals and humans show that mildly or moderately elevated homocysteine is associated with several important components of atherogenesis, including vascular inflammation, endothelial dysfunction, and hypercoagulability[4]; however, the extent to which these and other pathogenic mechanisms may underlie the apparent association with CVD remains unclear.

The WAFACS (Women's Antioxidant and Folic Acid Cardiovascular Study), conducted among women at high CVD risk, showed that combined treatment with folic acid, vitamin B_6, and vitamin B_{12} for 7.3 years significantly reduced homocysteine concentrations, but did not reduce a combined end point of total cardiovascular events.[5] Meta-analyses of other randomized trials in high-risk patients similarly indicate that homocysteine lowering with B vitamins has no beneficial effect on CVD or venous thrombosis, although a possible

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Clinical Perspective

What Is New?

- Our substudy findings from a large, randomized trial of women at increased risk of cardiovascular disease indicate that combined treatment with B vitamins for treatment durations as long as 7.3 years has no effect on plasma biomarkers of inflammation and endothelial function, despite a significant reduction in homocysteine concentration.

What Are the Clinical Implications?

- These findings may partly explain the absence of clinical benefit for homocysteine lowering in secondary prevention trials of cardiovascular disease.
- These findings also suggest that mildly or moderately elevated homocysteine may not be an important causal factor in vascular inflammation in patients at high risk for cardiovascular disease.

benefit on stroke has been suggested.[6–10] One interpretation of the mostly null findings in trials is that elevated homocysteine is a marker of suboptimal B-vitamin status, but is not causally related to CVD. Others have speculated that the beneficial effects of homocysteine lowering may be countered by adverse effects of high-dose B vitamins such as an increase in inflammation or endothelial dysfunction.[11,12] With regard to this possibility, current evidence, including substudies of randomized trials, suggests that homocysteine lowering with B vitamins has little impact on plasma biomarkers of inflammation[12–27] and endothelial dysfunction.[12,17,18,24,28,29] However, most of these studies have been limited by small sample size (n<200) and short treatment duration (<2 years).

To address this question, we investigated the effect of long-term, combined treatment with folic acid, vitamin B_6, and vitamin B_{12} on plasma biomarkers of chronic inflammation and endothelial dysfunction in a subsample of 300 participants in WAFACS.[5]

Methods

The data, analytical methods, and study materials will not be made available to other researchers for purposes of reproducing the results or replicating the procedure.

Study Population

The WAFACS was a randomized, double-blind, placebo-controlled trial that evaluated whether a daily combination of folic acid (2.5 mg), vitamin B_6 (50 mg), and vitamin B_{12} (1 mg) could reduce cardiovascular events among women with pre-existing CVD or 3 or more coronary risk factors. The WAFACS trial began in 1998 when the folic acid/B_6/B_{12} arm was added to the ongoing WACS (Women's Antioxidant Cardiovascular Study). The WACS was a $2\times2\times2$ factorial trial of 8171 women at high risk of CVD randomized between June 1995 and October 1996 to high-dose antioxidants (vitamin E, vitamin C, and beta carotene) or placebo. In April, 1998, 5442 of the 8171 WACS participants who were willing and eligible for participation in the new arm of the trial were further randomized to folic acid/B_6/B_{12} or placebo in a retained factorial design. Randomized treatment in the WAFACS ended on July 31, 2005 for a mean treatment duration of 7.3 years. Details of the overall trial design and the main results from the WAFACS and WACS have been reported previously.[5,30,31] The trial was approved by the institutional review board of the Brigham and Women's Hospital (Boston, MA), and all patients provided written informed consent.

Blood Substudy

A total of 2596 women in the WAFACS provided a baseline blood sample at study entry in 1996, preceding the initiation of background dietary folic acid fortification of the US food supply in 1998. From this group, 300 randomly selected participants who were adherent with study medications (150 in the active treatment group, 150 in the placebo group) provided a follow-up blood sample at the end of randomized treatment.

Blood Collection and Storage

Similar kits and procedures were used for baseline and follow-up blood collections in this preplanned substudy. Women were mailed a blood collection kit that contained instructions, three 10-mL EDTA vacutainer tubes, three 4.5-mL sodium citrate tubes, supplies needed to draw a sample of blood, a completed overnight courier air bill, and a gel-filled freezer pack. Women were asked to freeze the gel-filled freezer pack to serve as a coolant for mailing. The next day, they had a blood sample drawn into 2 EDTA and 2 citrate tubes and returned the completed kit by overnight courier. All samples arrived in our laboratory within 24 to 30 hours of venipuncture, were kept chilled until processed, and were stored at −120 to −160°C within 30 to 36 hours of venipuncture.

Measurement of Plasma Biomarkers

High-sensitivity C-reactive protein

The concentration of CRP (C-reactive protein) was determined using an immunoturbidimetric assay on the Hitachi 917 analyzer (Roche Diagnostics, Indianapolis, IN), using reagents and calibrators from DiaSorin (Stillwater, MN). This assay has a sensitivity of 0.03 mg/L. Interassay coefficients of variation (CVs) at concentrations of 0.91, 3.07, and 13.38 mg/L are 2.81%, 1.61%, and 1.1%, respectively.

Interleukin-6

Interleukin (IL-6) was measured by an ultrasensitive ELISA assay from R & D Systems (Minneapolis, MN) using the quantitative sandwich enzyme immunoassay technique. The assay has a sensitivity of 0.094 pg/mL, and interassay CVs at concentrations of 0.49, 2.78, and 5.65 pg/mL are 9.6%, 7.2%, and 6.5%, respectively.

Intracellular adhesion molecule-1

Intracellular adhesion molecule-1 (ICAM-1) was measured by an ELISA assay (R & D Systems) using the quantitative sandwich enzyme immunoassay technique. The assay has a sensitivity of 0.35 ng/mL, and interassay CVs at concentrations of 64.2, 117, 290, and 453 ng/mL are 10.1%, 7.4%, 6.0%, and 6.1%, respectively.

Fibrinogen

Concentration of fibrinogen was determined using an immunoturbidimetric assay on the Hitachi 917 analyzer (Roche Diagnostics), using reagents and calibrators from Kamiya Biomedical Co. Interassay CVs at concentrations of 167.4, 323.6, and 554.1 mg/dL are 0.94%, 1.06%, and 1.50%, respectively.

Folate

Concentration of folate was determined by a chemiluminescence method using the 2010 Elecsys utoimmunoanalyzer (Roche Diagnostics, Basel, Switzerland). Interassay CV ranges from 1.9% to 7.7% depending on plasma level.

Homocysteine

Concentration of homocysteine was determined using an enzymatic assay on the Hitachi 917 analyzer (Roche Diagnostics), using reagents and calibrators from Catch Inc. (Seattle, WA). Interassay CV ranges from 3.9% to 7.5% depending on plasma level.

Statistical Analyses

Baseline demographic and lifestyle characteristics in the 2 randomized treatment groups were compared using the Student *t* test for continuous variables expressed as means (SD) and chi-square tests for categorical data. Raw distributions and median values (interquartile range) of biomarkers at baseline were compared using the nonparametric Wilcoxon rank-sum test. Spearman correlation coefficients were used to examine the inter-relation of biomarkers in the combined sample at baseline.

We examined the effect of folic acid fortification in the United States in 1998 by determining the change in biomarker concentrations from baseline to follow-up in the placebo group. For folate, we compared the raw distributions and median values at baseline and follow-up using the nonparametric Wilcoxon signed-rank test. For homocysteine, CRP, ICAM-1, IL-6, and fibrinogen, we compared geometric means after natural logarithmic transformation at baseline and follow-up using the paired *t* test.

To assess the effect of treatment on plasma biomarkers, we compared the change from baseline to follow-up in both the active and placebo groups. For folate, we compared the change in ordinal categories in the active and placebo group using a repeated-measures cumulative logit model in PROC GENMOD of SAS. For homocysteine, CRP, ICAM-1, IL-6, and fibrinogen, we compared the change observed in the active group over the placebo group by computing the difference between groups in the change in the natural logarithm of biomarker concentration from baseline to follow-up.

Finally, because hormone replacement therapy (HRT) has been shown to increase plasma CRP concentrations, but reduce concentrations of all other biomarkers of vascular inflammation,[32,33] we examined the association at baseline of current HRT use and biomarker concentration, and whether change in concentration at the end of follow-up was associated with change in HRT use during follow-up.

Results

Table 1 displays the baseline characteristics of the 300 adherent participants in the blood substudy according to treatment group. Participants were similar with respect to all characteristics listed in the table except for history of diabetes mellitus (21.3% in the active treatment group versus 12.0% in the placebo group; P=0.03). Median plasma biomarker concentrations were similar in the active treatment and placebo groups at baseline (all $P \geq 0.20$).

Correlation Among Biomarkers at Baseline

Results of correlational analyses of the combined sample at baseline are presented in Table 2. Plasma folate showed a significant inverse correlation with homocysteine ($\rho=-0.29$; $P<0.0001$) and weaker inverse correlations with IL-6 ($\rho=-0.16$; $P<0.01$), fibrinogen ($\rho=-0.13$; $P<0.05$), and ICAM-1 ($\rho=-0.12$; $P<0.05$). Homocysteine was weakly correlated with ICAM-1 and fibrinogen (both $\rho=0.16$; $P<0.01$), but not with IL-6 or CRP. IL-6, ICAM-1, fibrinogen, and CRP were all positively intercorrelated (ranging from $\rho=0.28$ to 0.42; all $P<0.0001$).

Effect of Combination Treatment With Folic Acid, Vitamin B_6, and Vitamin B_{12} on Biomarkers

Folate and homocysteine

Table 3 displays the distribution of folate concentrations at baseline and follow-up for 150 participants in the placebo

Table 1. Baseline Characteristics According to Randomized Treatment Assignment

	Folic Acid/B_6/B_{12} (n=150)	Placebo (n=150)	*P* Value
Age (mean [SD] y)	62.1 (8.2)	62.1 (8.6)	0.94
Cigarette smoking, %			0.65
Never	55.3	50.0	
Former	37.3	41.3	
Current	7.3	8.7	
Alcohol use, %			0.49
Rarely/never	52.7	48.7	
≥1 drink/month	47.3	51.3	
BMI (mean [SD], kg/m^2)	30.7 (6.2)	30.1 (6.3)	0.44
Exercise ≥1 time/week, %	47.3	36.7	0.06
Reported history			
Hypertension*	86.0	88.0	0.61
High cholesterol†	76.0	81.3	0.26
Diabetes mellitus	21.3	12.0	0.03
CVD	65.3	56.7	0.12
Aspirin use‡, %	54.7	48.7	0.30
HRT, %	54.0	54.7	0.91
Current multivitamin use§, %	20.0	24.0	0.40
Randomized to vitamin E	44.7	48.7	0.49
Randomized to vitamin C	52.7	45.3	0.20
Randomized to beta carotene	57.3	55.3	0.73
Folate (median [IQR], ng/mL)	8.8 (6.4–12.8)	8.9 (6.0–13.4)	0.94
Homocysteine (median [IQR], μmol/L)	12.1 (10.2–15.0)	12.5 (9.6–15.5)	0.96
hsCRP (median [IQR], mg/L)	3.8 (1.8–7.4)	4.7 (1.9–8.7)	0.32
ICAM-1 (median [IQR], ng/mL)	260.1 (228.9–298.6)	268.4 (232.5–304.4)	0.57
IL-6 (median [IQR], pg/mL)	1.4 (0.9–1.9)	1.5 (1.1–2.2)	0.20
Fibrinogen (median [IQR], mg/dL)	486.4 (426.8–557.5)	501.1 (441.0–583.5)	0.22

BMI indicates body mass index; CVD, cardiovascular disease; HRT, hormone replacement therapy; hsCRP, high-sensitivity C-reactive protein; ICAM-1, intracellular adhesion molecule-1; IL-6, interleukin-6; IQR, interquartile range; WAFACS, Women's Antioxidant and Folic Acid Cardiovascular Study.

*Self-reported systolic blood pressure >140 mm Hg, diastolic blood pressure >90 mm Hg; self-reported physician-diagnosed hypertension; or reported treatment with medication for hypertension.

†Self-reported high cholesterol, cholesterol level >240 mg/dL; self-reported physician diagnosed, high cholesterol levels; or reported treatment with cholesterol-lowering medication.

‡Aspirin use at least 4 times per month.

§Any multivitamin use in the past month.

group and 150 participants in the active group. At baseline, before the initiation of fortification, folate concentrations were similar in the active treatment group and placebo group (median, 8.8 versus 8.9 ng/mL, respectively; *P*=0.94, data in Table 1), with approximately one third of participants in each group having concentrations considered inadequate (<7 ng/mL). At the end of study follow-up, median folate concentration increased significantly in the placebo group to 15.4 ng/mL (interquartile range, 11.5–22.6 ng/mL; *P*<0.001); however, the relative increase in folate concentration was greater in the active treatment group, in which 49.3% of participants had a folate concentration greater than 40 ng/mL (the upper limit of the assay) as compared with 4.7% in the placebo group (*P*<0.001).

Despite the significant increase in folate concentration in the placebo group, there was no apparent reduction in homocysteine in that group at the end of the study as compared with baseline (Table 4). In the active treatment group, homocysteine at the end of the study decreased by 18.3% compared with baseline, and this change was significantly greater than the change observed in the placebo group (*P*<0.001).

Table 2. Spearman Correlation Matrix for Baseline Plasma Concentrations of Folate, Homocysteine, and Biomarkers of Inflammation and Endothelial Dysfunction Among Blood Substudy Participants in WAFACS (n=300)

	Folate	Homocysteine	CRP	ICAM-1	IL-6	Fibrinogen
Folate	1.0					
Homocysteine	−0.29*	1.0				
CRP	−0.11	−0.09	1.0			
ICAM-1	−0.12†	0.16‡	0.29*	1.0		
IL-6	−0.16‡	0.10	0.41*	0.39*	1.0	
Fibrinogen	−0.13†	0.16‡	0.42*	0.28*	0.39*	1.0

CRP indicates C-reactive protein; ICAM-1, intracellular adhesion molecule-1; IL-6, interleukin-6; WAFACS, Women's Antioxidant and Folic Acid Cardiovascular Study.
*$P<0.0001$.
†$P<0.05$.
‡$P<0.01$.

CRP, IL-6, ICAM-1, and fibrinogen

As shown in Table 4, CRP concentrations in the placebo group decreased significantly ($P<0.001$) from baseline, whereas IL-6 increased significantly ($P<0.001$). ICAM-1 and fibrinogen at follow-up were not significantly different from baseline in the placebo group. For all 4 biomarkers, the changes observed in the active treatment group closely mirrored those in the placebo group, and there was no significant difference between treatment groups in change from baseline for any biomarker.

We examined whether the significant changes in CRP and IL-6 in both treatment groups were related to change in use of HRT during follow-up. At baseline, 163 participants reported current use of HRT. These participants had higher CRP concentrations ($P<0.001$) and lower concentrations of fibrinogen ($P<0.001$) and homocysteine ($P<0.001$) than did HRT nonusers (data not shown). During follow-up, 136 of the 163 HRT users discontinued use. As shown in Table 5, participants who discontinued HRT use during follow-up (ie, switchers) experienced a significant decrease in CRP concentration in both the placebo and active group. Participants who maintained HRT use during follow-up (ie, nonswitchers) experienced no change in CRP. For IL-6 and the other biomarkers, there was no clear relation between change in HRT use during follow-up and change in biomarker concentrations at the end of follow-up.

Discussion

These findings from a substudy of adherent participants in WAFACS indicate that combined treatment with folic acid, vitamin B_6, and vitamin B_{12} for 7.3 years significantly reduced plasma concentrations of homocysteine, but did not alter inflammatory responses involving CRP, IL-6, and fibrinogen or indices of endothelial dysfunction as reflected by ICAM-1 concentration. These findings may partly explain the null results in secondary prevention trials investigating the effect of homocysteine lowering on CVD events, including the WAFACS. This suggests that mildly or moderately elevated homocysteine may not be an important causal factor in vascular inflammation in patients at high risk for CVD.

Our findings for CRP, IL-6, and fibrinogen appear consistent with the null findings for these biomarkers in other trials

Table 3. Distribution of Plasma Folate Concentrations at Baseline and End of Treatment and Follow-Up Among Blood Substudy Participants in WAFACS

	Baseline, N (%)		Follow-up, N (%)	
Folate, ng/mL	Placebo (n=150)	Folic Acid/B_6/B_{12} (n=150)	Placebo (n=150)	Folic Acid/B_6/B_{12} (n=150)
<7	52 (34.7)	49 (32.7)	2 (1.33)	0
7 to <15	71 (47.3)	80 (53.3)	69 (46.0)	1 (0.67)
15 to <25	22 (14.7)	19 (12.7)	54 (36.0)	21 (14.0)
25 to 40	4 (2.67)	2 (1.33)	18 (12.0)	54 (36.0)
>40	1 (0.67)	0	7 (4.67)	74 (49.3)

WAFACS indicates Women's Antioxidant and Folic Acid Cardiovascular Study.

Table 4. Change in Biomarker Concentrations According to Treatment Group Among Blood Substudy Participants in WAFACS

	Placebo (n=150)				Folic Acid/B_6/B_{12} (n=150)				
	Baseline	Follow-Up	% Change*	*P* Value	Baseline	Follow-Up	% Change*	*P* Value	*P* Value†
Homocysteine (mean‡, μmol/L)	12.3	12.3	0.0	0.99	12.2	10.0	−18.3	<0.001	<0.001
CRP (mean‡, mg/dL)	3.9	2.5	−35.4	<0.001	3.5	2.4	−33.4	<0.001	0.77
ICAM-1 (mean‡, ng/mL)	266.1	259.2	−2.6	0.10	266.6	265.2	−0.5	0.77	0.38
IL-6 (mean‡, pg/mL)	1.5	1.8	23.8	<0.001	1.4	1.7	22.5	<0.001	0.91
Fibrinogen (mean‡, mg/dL)	501.7	519.0	3.5	0.053	486.9	508.3	4.4	<0.001	0.68

CRP indicates C-reactive protein; ICAM-1, intracellular adhesion molecule-1; IL-6, interleukin-6; WAFACS, Women's Antioxidant and Folic Acid Cardiovascular Study.
*Percent change in group mean.
†*P* value for difference in change in geometric mean from baseline to follow-up comparing treated and placebo groups.
‡Geometric mean.

of homocysteine lowering conducted in selected patient groups (eg, diabetes mellitus, coronary artery disease, and peripheral artery disease). These include 4 trials with treatment duration of 1 to 2 years, all of which reported no material impact of B-vitamin supplementation on plasma concentrations of CRP.[12,17,19,26] Nine other trials examined plasma concentrations of IL-6 or fibrinogen, and most,[13,14,16,18,21–24] but not all,[20] found no effect of B-vitamin supplementation on either biomarker. However, treatment duration in these trials was limited (6 months or less). Thus, our findings in WAFACS extend the previous null results for IL-6, fibrinogen, and CRP to treatment durations as long as 7.3 years. Taken together, these findings indicate that homocysteine lowering with long-term B vitamin treatment has no beneficial or harmful effect on plasma biomarkers of inflammation. This suggests that the association of elevated homocysteine with CVD is not likely to be explained by increased vascular inflammation.

We similarly found no beneficial or harmful effect of B-vitamin treatment on ICAM-1. ICAM-1 is often grouped with other inflammatory biomarkers (it is expressed on monocytes and contributes to inflammatory responses[34]), but is also a biomarker of endothelial dysfunction because it mediates the adherence and passage of leukocytes across the vascular wall early in atherogenesis. Several previous trials with treatment duration of up to 2 years also found no significant effect of B-vitamin supplementation on plasma concentration of this biomarker.[12,17,18,24,28,29] Our present findings extend these earlier null results for ICAM-1 to treatment durations of up to 7.3 years.

Consistent with the null findings for homocysteine lowering, our cross-sectional data at baseline also indicated little association between plasma homocysteine and the 4 biomarkers examined. These results are consistent with most,[12,14,21,35–39] but not all,[40] correlational findings for these biomarkers in other patient populations, and further support the conclusion that mildly or moderately elevated homocysteine is not an important causal factor in chronic vascular inflammation.

There was an ≈2-fold increase in plasma folate concentration, and almost complete elimination of inadequate folate concentrations (<7 ng/mL), at the end of study follow-up in the placebo group. These changes may reflect the impact of folic acid fortification of the US food supply in 1998, which was estimated to provide an additional 70 to 120 μg/day of folic acid among adults aged ≥50 years.[41] Similar magnitude changes in plasma folate concentrations following fortification were observed in other populations, including the Framingham Offspring Study.[42,43] The dose of folic acid tested in the WAFACS, 2.5 mg/day, exceeded the tolerable upper intake level for folic acid of 1 mg/day.[44] Consequently, approximately half of participants in the treated group had folate concentrations >40 ng/mL at the end of study follow-up, which is well above normal reference ranges for plasma folate.[45,46] Some have expressed concern that high folic acid concentrations may be proinflammatory and could perhaps counter any potential beneficial effect of folic acid supplementation.[11,12,47] However, we found no evidence that high-dose folic acid supplementation increased levels of inflammation as reflected by change in plasma concentration of any of the biomarkers examined. Moreover, we found no increased risk of chronic diseases associated with inflammation, including CVD, cancer, and diabetes mellitus, in WAFACS, nor was there an increase in all-cause mortality in that trial.[5,48,49]

The observation of a significant decrease in CRP, and increase in IL-6, in the placebo group was unexpected. In exploratory analyses, we found that discontinuation of HRT use during follow-up, perhaps in response to the disappointing results for HRT in the Women's Health Initiative in 2002,[50] likely contributed to the decrease in CRP concentrations observed in both the placebo and treated groups. However, the clinical significance of the increase in IL-6 in both groups is unclear, and discontinuation of HRT did not appear to

Table 5. Change in Biomarker Concentrations According to Treatment Group Among 163 HRT Users at Baseline (136 Switchers vs 27 Nonswitchers) and 137 HRT Nonusers at Baseline (45 Switchers vs 92 Nonswitchers) Among Blood Substudy Participants in WAFACS

	Placebo				Folic Acid/B_6/B_{12}				
	Baseline	Follow-Up	% Change	*P* Value	Baseline	Follow-Up	% Change	*P* Value	*P* Value*
HRT users at baseline (n=163)									
Nonswitchers	(n=10)				(n=17)				
Homocysteine (mean†, μmol/L)	10.68	11.17	4.6	0.80	12.33	9.15	−25.8	0.002	0.048
CRP (mean†, mg/dL)	3.94	3.70	−6.2	0.82	3.06	2.66	−12.9	0.46	0.82
ICAM-1 (mean†, ng/mL)	237.37	233.13	−1.8	0.73	245.85	225.18	−8.4	0.04	0.29
IL-6 (mean†, pg/mL)	1.19	1.77	49.5	0.003	1.03	1.32	28.6	0.10	0.47
Fibrinogen (mean†, mg/dL)	494.61	510.04	3.1	0.67	411.14	428.25	4.2	0.16	0.89
Switchers	(n=72)				(n=64)				
Homocysteine (mean†, μmol/L)	11.36	12.00	5.7	0.17	11.64	10.21	−12.3	0.004	0.002
CRP (mean†, mg/dL)	5.44	2.40	−55.8	<0.001	4.25	2.29	−46.0	<0.001	0.17
ICAM-1 (mean†, ng/mL)	268.68	261.54	−2.7	0.29	271.59	277.80	2.3	0.43	0.19
IL-6 (mean†, pg/mL)	1.47	1.77	20.9	0.001	1.43	1.75	22.7	0.009	0.87
Fibrinogen (mean†, mg/dL)	492.16	503.63	2.3	0.43	473.90	512.15	8.1	0.001	0.12
HRT nonusers at baseline (n=137)									
Nonswitchers	(n=42)				(n=45)				
Homocysteine (mean†, μmol/L)	14.81	13.30	−10.2	0.07	13.09	9.75	−25.5	<0.001	0.02
CRP (mean†, mg/dL)	3.41	3.03	−11.3	0.50	2.99	2.22	−25.6	0.005	0.39
ICAM-1 (mean†, ng/mL)	279.62	267.03	−4.5	0.14	270.77	273.14	0.9	0.82	0.27
IL-6 (mean†, pg/mL)	1.85	2.19	18.6	0.13	1.53	1.93	26.1	0.01	0.66
Fibrinogen (mean†, mg/dL)	536.77	549.95	2.5	0.41	522.53	537.19	2.8	0.26	0.93
Switchers	(n=26)				(n=24)				
Homocysteine (mean†, μmol/L)	11.92	11.99	0.6	0.93	12.28	10.55	−14.0	0.04	0.11
CRP (mean†, mg/dL)	2.00	1.92	−4.2	0.86	3.27	2.57	−21.4	0.18	0.51
ICAM-1 (mean†, ng/mL)	249.76	250.98	0.5	0.87	260.73	248.77	−4.6	0.06	0.17
IL-6 (mean†, pg/mL)	1.12	1.46	29.4	0.03	1.28	1.42	11.3	0.39	0.36
Fibrinogen (mean†, mg/dL)	476.82	517.18	8.5	0.01	516.99	506.85	−2.0	0.44	0.01

CRP indicates C-reactive protein; HRT, hormone replacement therapy; ICAM-1, intracellular adhesion molecule-1; IL-6, interleukin-6; WAFACS, Women's Antioxidant and Folic Acid Cardiovascular Study.

**P* value for difference in change in geometric mean from baseline to follow-up comparing treated and placebo groups.

†Geometric mean.

materially alter plasma concentrations of IL-6 or any of the other biomarkers examined.

Several potential limitations of this substudy warrant consideration. Measurement of plasma biomarkers may not accurately reflect biomarker status at the cellular level. We also cannot exclude effects on other biomarkers of inflammation or endothelial function that were not measured. Participants in the WAFACS were at high risk of CVD, so the results may not be directly generalizable to the general population. It is also possible that the results would be different in populations with higher homocysteine levels than the WAFACS. Finally, this substudy suggests that approximately one third of participants in the WAFACS were folate deficient at the beginning of the trial, but this was virtually eliminated over the course of the study, perhaps attributed to background folic acid fortification in 1998. Therefore, we cannot rule out the possibility that this same treatment regimen might have resulted in an even greater reduction in homocysteine, and a different impact on inflammatory and endothelial function biomarkers in a more folate-deficient, unfortified population.

In summary, these substudy findings from a large, randomized trial of women at increased risk of CVD indicate that long-term, combined treatment with folic acid, vitamin B_6,

and vitamin B_{12} has no effect on plasma biomarkers of inflammation and endothelial dysfunction, despite significant reductions in plasma homocysteine. This may partly explain the absence of clinical benefit for homocysteine-lowering in the WAFACS and other secondary prevention trials of CVD. The findings also appear consistent with a growing body of evidence that suggests that mildly or moderately elevated homocysteine may not be an important causal factor in vascular inflammation in patients at high risk for CVD, and that the association of homocysteine with CVD is unlikely to be attributed to chronic vascular inflammation.

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Disclosures

None.

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