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Biomarkers of One-Carbon Metabolism Are Associated with Biomarkers of Inflammation in Women[1–3]

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Abstract

Folate-mediated one-carbon metabolism is essential for DNA synthesis, repair, and methylation. Perturbations in one-carbon metabolism have been implicated in increased risk of some cancers and may also affect inflammatory processes. We investigated these interrelated pathways to understand their relation. The objective was to explore associations between inflammation and biomarkers of nutritional status and one-carbon metabolism. In a cross-sectional study in 1976 women selected from the Women's Health Initiative Observational Study, plasma vitamin B-6 [pyridoxal-5′-phosphate (PLP)], plasma vitamin B-12, plasma folate, and RBC folate were measured as nutritional biomarkers; serum C-reactive protein (CRP) and serum amyloid A (SAA) were measured as biomarkers of inflammation; and homocysteine and cysteine were measured as integrated biomarkers of one-carbon metabolism. Student's t, chi-square, and Spearman rank correlations, along with multiple linear regressions, were used to explore relations between biomarkers; additionally, we tested stratification by folic acid fortification period and multivitamin use. With the use of univariate analysis, plasma PLP was the only nutritional biomarker that was modestly significantly correlated with serum CRP and SAA ($\rho = -0.22$ and -0.12, respectively; $P < 0.0001$). Homocysteine (μmol/L) showed significant inverse correlations with all nutritional biomarkers (ranging from $\rho = -0.30$ to $\rho = -0.46$; all $P < 0.0001$). With the use of multiple linear regression, plasma PLP, RBC folate, homocysteine, and cysteine were identified as independent predictors of CRP; and PLP, vitamin B-12, RBC folate, and homocysteine were identified as predictors of SAA. When stratified by folic acid fortification period, nutrition-homocysteine correlations were generally weaker in the postfortification period, whereas associations between plasma PLP and serum CRP increased. Biomarkers of inflammation are associated with PLP, RBC folate, and homocysteine in women. The connection between the pathways needs to be further investigated and causality established. The trial is registered at clinicaltrials.gov as NCT00000611.   J. Nutr. 144: 714–721, 2014.

Introduction

The importance of folate-mediated one-carbon metabolism in colorectal carcinogenesis has been shown in numerous epidemiologic and animal studies (1–3). Folate functions as a donor of one-carbon units and is essential for methylation reactions, including DNA methylation, as well as nucleotide and DNA synthesis, stability, and repair (4,5). The universal and essential methyl donor, S-adenosylmethionine, is produced in the

folate-dependent homocysteine cycle, which includes the conversion from homocysteine to methionine by demethylation of 5′methyltetrahydrofolate.

Several B vitamins, including folate, vitamin B-12, and vitamin B-6, play important roles as substrates or cofactors in one-carbon metabolism. Deficiencies of these B vitamins disturb the pathway leading to the following: *1*) inhibited DNA synthesis and repair, *2*) decreased DNA stability, *3*) altered methylation patterns, and *4*) hyperhomocysteinemia. Such disturbances in one-carbon metabolism are thought to contribute to cancer, particularly colorectal cancer (6), as well as cardiovascular disease and other metabolic and psychiatric disorders such as depression, dementia, and Alzheimer disease (7–11).

In recent years, there has been growing recognition that chronic inflammation is an important pathogenic factor underlying degenerative diseases, including cancer and cardiovascular disease (12). Moreover, there are increasing numbers of reports of associations between B vitamins, homocysteine, and inflammatory biomarkers, suggesting important interrelations between perturbations in one-carbon metabolism and inflammatory reactions. For example, patients with rheumatoid arthritis, diabetes, or stroke, conditions in which inflammation plays a crucial role, showed inverse associations between plasma pyridoxal-5′-phosphate (PLP)[13] and the inflammatory marker C-reactive protein (CRP) in serum (13–18). Increased concentrations of the acute inflammatory marker serum amyloid A (SAA) can be found in patients with acute and chronic inflammation (19). Inflammation can result in cell damage and proliferation, and has been directly implicated in risk of carcinogenesis (e.g., through reactive oxygen species), causing damage to macromolecules, including DNA (20,21).

To further define the interaction between the 2 biological pathways, one-carbon metabolism and inflammation, we investigated the association between the following: *1*) nutritional biomarkers plasma PLP, vitamin B-12, plasma folate, and RBC folate; *2*) biomarkers of inflammation (serum CRP and SAA); and *3*) one-carbon metabolism (homocysteine and cysteine concentrations) in the Women's Health Initiative Observational Study (WHI-OS) (22). A concurrent investigation of the interrelated pathways may yield new information regarding the relation. Depending on the status of folic acid fortification, the strength of the association may differ, but this has not yet been investigated previously.

The study presented here is largely exploratory, with only a limited number of set hypotheses. Specifically, we expected low plasma PLP status to be associated with high serum CRP and high SAA concentrations and high folate concentrations to be associated with decreased serum CRP concentrations. Furthermore, we hypothesized that high homocysteine and low cysteine concentrations would be associated with high serum CRP.

Participants and Methods

Study population
A cross-sectional study design was used to explore correlations between biomarkers of nutrition, inflammation, and one-carbon metabolism in 1976 women selected from the 93,676 participants in the WHI-OS, as part of a nested case-control study (23). All biomarkers were measured at baseline in participants without clinical disease. The WHI-OS enrolled participants at 40 U.S. clinical institutions between 1993 and 1998. The study design and characteristics of the cohort have been described elsewhere (22,24). Eligibility requirements included postmenopausal

status, age between 50 and 79 y, and low likelihood of loss to follow-up within 3 y due to relocation or death resulting from a preexisting medical condition. We subdivided participants into 3 groups reflecting time of recruitment in relation to folic acid fortification in the United States, i.e., prefortification (before 1996), perifortification (1996–1997), and postfortification (1998 and later) phases.

All biomarkers, including plasma PLP, plasma vitamin B-12, plasma folate, RBC folate, serum CRP, SAA, plasma homocysteine, and plasma cysteine, were measured by using fasting blood samples collected at recruitment into the cohort. Of the 1976 participants, 988 were selected because they later developed colorectal cancer, and 988 were controls matched by age (±3 y), race/ethnicity, clinical center, date of blood draw (±6 mo for baseline and year 3 blood draws), and baseline hysterectomy status. For the analysis, women were excluded if they had preexisting intestinal disease, including history of colorectal cancer ($n = 959$), carcinoma in situ ($n = 46$), ulcerative colitis, Crohn disease, or if they were extremely under- or overweight as indicated by a BMI ≤15 or ≥50 kg/m^2, respectively ($n = 502$). We excluded women with cancer occurring within 1 y after recruitment ($n = 184$) (**Fig. 1**). To further address the potential that some of the women had preclinical disease, in our analyses we excluded cases diagnosed within a year of baseline. The study was approved by the human subjects review boards at the WHI-OS Clinical Coordinating Center at the Fred Hutchinson Cancer Research Center, at all 40 clinical centers at which participants were recruited for the WHI-OS study, and at the University of California, Davis, and by the ethics commission of the University of Heidelberg. Written informed consent was obtained from all participants.

Demographic and health data collection
Demographic characteristics, including age, race, and education, were collected by using standardized instruments. Height and weight were measured at baseline by trained staff with the use of a common protocol, and BMI was calculated by using the equation BMI = weight (kg)/height (m^2).

Sample processing and analysis
For the purpose of measuring the concentrations of the study-related biomarkers, a baseline blood sample was collected. Participants were instructed to fast at least 12 h before phlebotomy. Blood samples were kept at 4°C for up to 1 h before centrifugation. Plasma and serum were collected and stored at −70°C in a central biorepository until analysis.
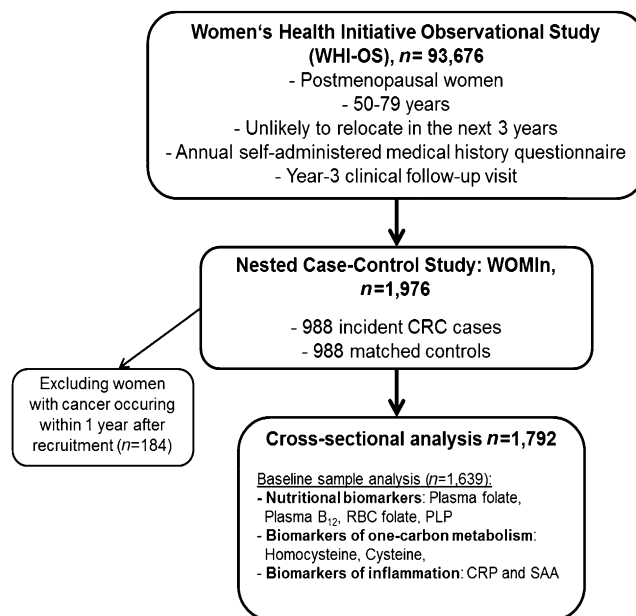

Women's Health Initiative Observational Study (WHI-OS), *n*= 93,676
- Postmenopausal women
- 50-79 years
- Unlikely to relocate in the next 3 years
- Annual self-administered medical history questionnaire
- Year-3 clinical follow-up visit

Nested Case-Control Study: WOMIn, *n*=1,976
- 988 incident CRC cases
- 988 matched controls

Excluding women with cancer occuring within 1 year after recruitment (*n*=184)

Cross-sectional analysis *n*=1,792
Baseline sample analysis (*n*=1,639):
- **Nutritional biomarkers**: Plasma folate, Plasma B$_{12}$, RBC folate, PLP
- **Biomarkers of one-carbon metabolism**: Homocysteine, Cysteine,
- **Biomarkers of inflammation**: CRP and SAA

FIGURE 1 Flow chart of the study populations. CRC, colorectal cancer; CRP, C-reactive protein; PLP, pyridoxal-5′-phosphate; SAA, serum amyloid A; WOMIn, Women, One-Carbon Metabolism and Inflammation Study.

Nutritional biomarkers. Plasma folate and plasma vitamin B-12 were determined by radioassay (SimulTRAC B$_{12}$/ FOLATE-SNB 57_Co/^{125}I; MP Biomedicals) at the Biomarker Laboratory, Fred Hutchinson Cancer Research Center (X.S.), RBC folate was measured by radioassay (SimulTRAC; MP Biomedicals), and plasma PLP was measured by HPLC with fluorescence detection at the University of California, Davis (J.W.M.) (25). Interassay CVs for WHI-OS blind duplicate samples were as follows: RBC folate, 10.2%; plasma folate, 4.8%; vitamin B-12, 6.2% and plasma PLP, 4.8% (25).

One-carbon status markers. Total plasma homocysteine and plasma cysteine were determined by HPLC with postcolumn fluorescence detection (26). Interassay CVs for the assays were as follows: homocysteine, 6.5%; cysteine, 7.1% (measured at the University of California, Davis; J.W.M.).

Inflammation markers. Serum CRP and SAA were quantified at the Clinical Immunology Laboratory (University of Washington) by latex-enhanced nephelometry (BNII; Siemens) (M.H.W.). CVs for WHI-OS blind duplicate samples were 4.9% for SAA and 4.1% for CRP. Because renal function is a strong determinant of homocysteine and cysteine concentrations, all correlations including these biomarkers were adjusted for creatinine. Plasma creatinine was determined by the Jaffe rate reaction method (DxC Instrument; Beckman Coulter), with a CV of 4.1%.

Statistical analyses

Descriptive statistics were assessed for baseline characteristics of all participants. Means and SDs for selected biomarkers were calculated. All analyzed biomarkers were evaluated by using scatter plots and distribution tables. Unadjusted and adjusted Spearman rank correlation coefficients and corresponding P values for the study population were calculated. Pathway analyses were considered to visualize the strength of associations between a pair of biomarkers while taking into account the impact of other biomarkers in the pathway. The adjusted correlations were controlled for baseline BMI, age, and plasma creatinine. Additionally, plasma homocysteine and plasma cysteine were adjusted for plasma vitamin B-12, plasma folate, and the combination of both, due to the dependency on these nutrients in the homocysteine metabolic pathway. Correlation analyses were also stratified by BMI (<25, 25–30, >30–35, >35 kg/m^2), age (<67, ≥67 y), fortification period [prefortification (1994–1995), perifortification (1996–1997), and postfortification (1998)], and multivitamin use ("currently taking" multivitamins with minerals: yes or no) (27). Univariate and multiple linear regression analyses were used to determine relations between the nutritional and one-carbon biomarkers with biomarkers of inflammation. Nutritional biomarkers included plasma vitamin B-6, plasma vitamin B-12, and RBC folate; one-carbon biomarkers included homocysteine and cysteine. All linear models were controlled for baseline BMI and age. In the multivariate analyses, all factors were included in the model and adjusted for age and BMI. Log transformations were performed on the inflammatory biomarkers of interest (serum CRP and SAA) to improve the normality of the distributions and to meet the required assumptions of the model building. Significance was defined as $P < 0.05$, and all statistical tests were 2-sided. Pathway analyses were conducted with R package *qgraph* (R Foundation), and all other analyses were conducted with SAS 9.3 (SAS Institute) (28).

Results

Baseline characteristics for all study participants (n = 1792) are shown in **Table 1**. Mean age was 67 y, mean BMI was 28.0 kg/m^2, and the majority of participants were Caucasian. Nearly 40% of participants took multivitamins, and 75% used nonsteroidal anti-inflammatory drugs at the time of recruitment.

Spearman correlations between nutritional biomarkers, integrated markers of one-carbon status, and inflammatory biomarkers are presented in **Table 2**. A visual representation of the correlation matrices is given in **Figure 2**.

We evaluated the nutritional markers, and plasma PLP showed significant positive correlations with all of the other nutritional

TABLE 1 Baseline characteristics of the study population[1]

Characteristic	Value
Age, y	67 ± 7 (1792)
BMI, kg/m^2	28 ± 6 (1773)
Ethnicity (n = 1792), n (%)	
White	1538 (86)
Black or African American	144 (8)
Other (Hispanic, Asian or Pacific Islander, American Indian or Alaskan Native, other, missing)	110 (6)
Residence location (U.S. region) (n = 1792), n (%)	
Northeast	434 (24)
South	430 (24)
Midwest	410 (23)
West	518 (29)
Education, n (%)	
≤High school	375 (21)
≥College	1403 (79)
Taking any multivitamin, n (%)	
Yes	701 (39)
No	1091 (61)
Taking multivitamin without minerals, n (%)	
Yes	80 (4)
No	1712 (96)
Taking multivitamin with minerals, n (%)	
Yes	626 (35)
No	1166 (65)
NSAID use, n (%)	1343 (75)
Pack-years of smoking	10.8 ± 19.6 (1730)
Alcohol use (12 drinks ever), n (%)	1577 (89)
Biomarker	
Plasma PLP, nmol/L	97.5 ± 96.7
Plasma vitamin B-12, pg/mL	535 ± 274
RBC folate, μg/L	600 ± 254
Plasma folate, μg/L	19.5 ± 14.0
Serum CRP, g/L	4.6 ± 6.2
SAA, g/L	9.7 ± 22.3
Plasma homocysteine, μmol/L	8.6 ± 3.1
Plasma cysteine, μmol/L	285 ± 39.5

[1] Values are means ± SDs (n) unless otherwise indicated. CRP, C-reactive protein; NSAID, nonsteroidal anti-inflammatory drug; PLP, pyridoxal-5′-phosphate; SAA, serum amyloid A.

markers, i.e., with plasma folate (ρ = 0.49, $P < 0.0001$), RBC folate (ρ = 0.41, $P < 0.0001$), and vitamin B-12 (ρ = 0.45, $P < 0.0001$). Furthermore, plasma folate was strongly correlated with RBC folate (ρ = 0.59, $P < 0.0001$) and moderately with vitamin B-12 (ρ = 0.40, $P < 0.0001$); RBC folate was also correlated with vitamin B-12 (ρ = 0.38, $P < 0.0001$).

With regard to the markers of one-carbon metabolism and their association with nutritional biomarkers, homocysteine was inversely correlated with all nutritional biomarkers measured in this study [ranging in correlation coefficients from ρ = −0.46 ($P < 0.0001$) for vitamin B-12 to ρ = −0.30 ($P < 0.0001$) for plasma folate adjusted for plasma vitamin B-12]. Cysteine was moderately positively correlated with plasma PLP (ρ = 0.13, $P < 0.0001$) and plasma folate (ρ = 0.11, $P = 0.002$).

We investigated the inflammatory biomarkers serum CRP and SAA as outcomes in univariate models, and plasma PLP was the only nutritional one-carbon metabolism biomarker to show a modest inverse correlation with both serum CRP and SAA. Although homocysteine was not significantly correlated with serum CRP and SAA, cysteine showed significant inverse

TABLE 2 Spearman correlation matrix of nutritional, one-carbon status, and inflammation biomarkers in postmenopausal women, adjusted for urine creatinine, age, and BMI[1]

	Nutritional biomarkers					Integrated markers of one-carbon status				Inflammation biomarkers	
	Plasma PLP	Plasma folate	Plasma folate[2]	RBC folate	Plasma vitamin B-12	Plasma Hcy	Plasma Hcy[3]	Plasma Cys	Plasma Cys[3]	Serum CRP	SAA
Nutritional biomarkers											
Plasma PLP		0.49***	0.38***	0.41***	0.45***	−0.34***	−0.05	0.13***	0.10**	−0.22***	−0.12***
Plasma folate				0.59***	0.40***	−0.43***	N/A	0.11***	N/A	0.01	0.03
Plasma folate[2]				0.51***	N/A	−0.30***	N/A	0.11***	N/A	0.001	0.02
RBC folate					0.38***	−0.37***	−0.10***	0.05	−0.008	0.05	0.06
Plasma vitamin B-12						−0.46***	N/A	0.03	N/A	0.03	0.03
Integrated markers of one-carbon status											
Plasma Hcy								0.38***	0.50***	0.003	0.03
Plasma Hcy[3]								0.50***	0.50***	0.02	0.06
Plasma Cys										−0.10***	−0.02
Plasma Cys[3]										−0.10***	−0.02
Inflammation biomarkers											
Serum CRP											0.46***

[1] ** P < 0.01, *** P < 0.001. CRP, C-reactive protein; Cys, cysteine; Hcy, homocysteine; N/A, not applicable; PLP, pyridoxal-5′-phosphate; SAA, serum amyloid A.
[2] Adjusted for plasma vitamin B-12.
[3] Adjusted for plasma folate and plasma vitamin B-12.

correlations with serum CRP ($\rho = -0.10$, $P = 0.002$), although not with SAA.

The stratified results by BMI and by multivitamin use showed no differences in correlation (**Supplemental Table 1**) When stratifying by fortification period (**Table 3**), the strengths of correlations between plasma PLP and homocysteine, cysteine, RBC folate, and plasma folate decreased from the prefortification to the postfortification period. We additionally stratified the results by serum CRP ($\leq$, >3 mg/L) and homocysteine ($\leq$, >12 μmol/L) (**Supplemental Tables 2** and **3**). The stratification by serum CRP showed differences in the correlation strength between plasma PLP and plasma vitamin B-12 ($P = 0.06$), with a stronger correlation in the serum CRP $\leq$3 mg/L category.

However, the correlation between the nutritional biomarker plasma PLP and the inflammatory marker serum CRP increased in strength from $\rho = -0.17$ prefortification to $\rho = -0.33$ postfortification (prefortification $\times$ postfortification P-interaction = 0.07). Correlations were attenuated between *1*) plasma and RBC folate, *2*) plasma folate and vitamin B-12, and *3*)plasma folate and homocysteine, across the periods with increasing folic acid fortification.

Linear regression analyses were performed, regressing nutritional and one-carbon metabolism biomarkers in 1 model predicting the inflammatory biomarkers serum CRP (mg/L) and SAA (mg/L), controlling jointly for age and BMI (**Table 4**). Significant results similar to the correlation analysis were observed when serum CRP (mg/L) and SAA (mg/L) were regressed on plasma PLP (nmol/L) (adjusted regression coefficient $\beta = -1.46$ and -1.04, respectively; $P < 0.0001$), after adjusting for all biomarkers. Furthermore, there were significant increases in log CRP by 0.32 mg/L ($P < 0.0001$) and log SAA by 0.25 mg/L ($P = 0.001$) for every 1-g/L increase in RBC folate. Also, vitamin B-12 (pg/mL) was inversely significantly associated with SAA (β: 0.23, $P = 0.002$), but not with CRP, in these multivariate models. Last, homocysteine (μmol/L) was significantly associated with both serum CRP (β: 21.04, $P = 0.002$) and SAA (β: 26.61, $P < 0.0001$). Cysteine (μmol/L) was significantly associated with serum CRP but not SAA.

Discussion

In this cross-sectional study we explored the associations between nutritional biomarkers, integrated markers of one-carbon metabolism, and biomarkers of inflammation among women enrolled in the WHI-OS.

As expected, all nutritional markers interrcorrelated positively. Furthermore, as expected from previous studies, homocysteine was inversely correlated with all nutritional biomarkers measured, because the compound is an integral part of the transmethylation and transsulfuration pathways, which are dependent on B vitamins.

An interesting finding is that plasma PLP was the only nutritional biomarker to show significant inverse correlations with both serum CRP and SAA in the univariate analysis; and importantly, this association remained consistent when stratifying by multivitamin use and grew stronger in the postfortification period. Furthermore, the multivariate analysis showed significant correlations for plasma PLP and the inflammatory markers. SAA and CRP are nonspecific hepatic inflammatory markers produced in response to infection, trauma, and other inflammatory states. In an acute phase, serum concentrations increase and slowly return to normal values within days, whereas persistent elevation occurs with chronic inflammatory states, including obesity (29,30). In other work within this study
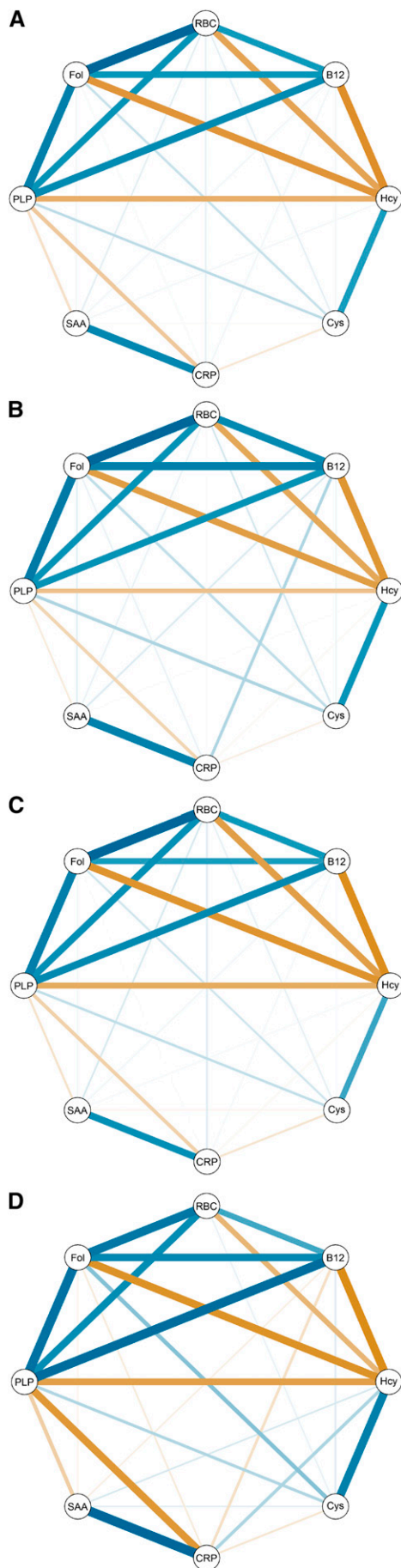
population, increased concentrations of CRP and SAA were associated with increased risk of developing colorectal cancer (23). Low plasma PLP concentrations could lead to an early inflammatory response, characterized by increased serum CRP and SAA concentrations. Interestingly, SAA concentrations predicted overall survival (more strongly than CRP) in a prospective study in 807 breast cancer patients (31). To our knowledge, no previous studies have investigated correlations between plasma PLP and SAA.

The inverse association between plasma PLP and serum CRP reported here has been noted in previous studies in healthy populations and in connection with disease conditions. For example, the Framingham Heart Study showed that low plasma PLP was associated with higher CRP concentrations when 891 participants were divided into normal and high baseline CRP concentrations (32). Higher PLP quartiles were significantly associated with lower CRP concentrations (P-trend < 0.0001) in the Boston Puerto Rican Health Study, with a strong dose-response relation (33). The 2003–2004 NHANES reported that, among 2686 participants, plasma PLP was inversely related to serum CRP, independent of dietary PLP intake ($P < 0.001$) (34). An additional population-based study in 1320 participants by Gori et al. (35) showed a similar association for serum CRP and plasma PLP in both men and women. Some other groups did not report an inverse relation between PLP and CRP. For example, a study on B-vitamin status and inflammatory markers recorded no associations between plasma PLP and CRP concentrations in 519 healthy middle-aged men and women (36). In summary, there is evidence for an inverse relation between PLP and CRP, but it is not entirely reproducible in all study populations. Our work extends this further to a novel association with SAA, and to stronger correlations in the post- vs. prefortification period, as discussed below.

Actual folate intake of the cohort has been published previously. In this population, the mean dietary folate intake was 234 μg/d and the mean supplemental folic acid intake was 204 μg/d. Dietary folate intake was associated with an increased colorectal cancer risk among women who had experienced the initiation of folic acid fortification (P-interaction < 0.01) (27).

Correlations between plasma folate, RBC folate, and plasma vitamin B-12 and the inflammatory markers were not significant in the univariate analysis. In the multivariate model, however, RBC folate showed a significantly positive association with CRP and SAA, although less robust than the findings for PLP. This finding is surprising and may not have been observed previously, because other studies investigated folate biomarkers without adjusting for other biomarkers. Folic acid intake has increased in the United States since fortification started in 1996. Several studies have been conducted to clarify a potential link between folic acid supplementation and disease. Meta-analyses evaluating the effects of folic acid supplementation on cancer incidence and cardiovascular disease showed no significant association, with the exception of a slight beneficial effect of folic acid in stroke prevention (37,38). Not all of the studies in the meta-analysis

FIGURE 2 (*A*) Pathway analysis in the spring format for nutritional, inflammatory, and integrated biomarkers of one-carbon metabolism. All correlations are partial between each pair of nodes (adjusted for baseline BMI, age). Blue indicates a positive correlation and orange indicates a negative correlation. The weight of the line indicates the strength of the correlation. Pathway analyses for the prefortification (*B*), perifortification (*C*), and postfortification (*D*) periods. B12, plasma vitamin B-12; CRP, serum C-reactive protein; Cys, plasma cysteine; Fol, plasma folate; Hcy, plasma homocysteine; PLP, pyridoxal-5′-phosphate; SAA, serum amyloid A.

TABLE 3 Spearman correlation matrix of biomarkers in postmenopausal women, stratified by pre-, peri-, and postfortification status and adjusted for urine creatinine, age, and BMI[1]

	Plasma Hcy			Plasma Cys			Serum CRP			SAA			Plasma vitamin B-12			RBC folate			Plasma folate		
	Pre	Peri	Post	Pre	Peri	Post	Pre	Peri	Post	Pre	Peri	Post	Pre	Peri	Post	Pre	Peri	Post	Pre	Peri	Post
Plasma PLP	−0.26**	−0.37***	−0.31***	0.16**	0.13**	0.12	−0.17**	−0.20***	−0.33***[2]	−0.08	−0.12**	−0.16***	0.42***	0.48***	0.43***	0.41***	0.45***	0.34***	0.51***	0.52***	0.42***
Plasma folate	−0.41***	−0.47***	−0.35***	0.12*	0.10*	0.18***	0.05	0.007	−0.06	0.06	0.03	−0.04	0.49***	0.39***	0.35***	0.58***	0.62***	0.40***			
RBC folate	−0.37***	−0.42***	−0.24***	0.08	0.05	0.04	0.007	0.07	0.0001	0.05	0.08*	0.002	0.45***	0.41***	0.26***						
Plasma vitamin B-12	−0.44***	−0.50***	−0.37***	0.04	0.02	0.05	0.15*	0.03	−0.10	0.07	0.03	−0.05									
SAA	−0.006	0.02	0.06	0.005	−0.04	0.04	0.48***	0.45***	0.45***												
Serum CRP	−0.02	−0.03	0.12	−0.06	−0.11**	−0.08															
Plasma Cys	0.40***	0.36***	0.38***																		

[1] Fortification periods: recruitment prefortification before 1996, perifortification between 1996 and 1997, and postfortification in 1998. * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$. CRP, C-reactive protein; Cys, cysteine; Hcy, homocysteine; peri, perifortification; PLP, pyridoxal-5′-phosphate; post, postfortification; pre, prefortification; SAA, serum amyloid A.

[2] Two-tailed P-value between pre- and postfortification periods = 0.07.

concurred; some of them accounted for folic acid fortification in some of the populations, and some did not.

Although substantial efforts have been undertaken to identify a connection between folic acid fortification and disease, it seems that no studies have been published regarding associations between higher folate status and elevated inflammatory markers (CRP and SAA). It is a caveat, however, that the observed expected inverse associations between RBC folate and homocysteine do not fit with the observed positive associations of RBC folate with serum CRP and SAA. Overall, it seems to be an appropriate next step to analyze these general correlations further and evaluate biologic mechanisms. Along these lines, we note that the antifolate drug, methotrexate, is used to treat rheumatoid arthritis, suggesting the anti-inflammatory effect of the drug is triggered by the inhibition of dihydrofolate reductase and consequent diminishing of de novo synthesis of purines and pyrimidines (39). This is consistent with our finding that folate and inflammation are directly correlated.

There is continued debate over folic acid supplementation and health effects (40). We observed differences in associations depending on fortification period. The inverse correlations between plasma PLP and the inflammatory markers serum CRP and SAA were stronger postfortification compared with the prefortification period. A similar pattern was observed for plasma PLP and SAA. This is a novel finding, which led us to the hypothesis that with a higher availability of folate, more plasma PLP is necessary for reactions in one-carbon metabolism that are linked to inflammatory processes.

Although serum CRP has been described as a general inflammatory marker, homocysteine is known as a modest predictor for coronary heart disease, a condition known to be associated with a chronic inflammatory process (41). As expected, we observed a positive association between homocysteine and serum CRP in our study population in the multivariate analyses. The univariate analysis correlations were not significant. Homocysteine showed similar results with SAA; the univariate analysis was nonsignificant, whereas the multivariate analysis presented a strong correlation. Furthermore, the significant inverse association between cysteine and CRP in the univariate and the multivariate analysis is an interesting finding, because cysteine, a partially essential amino acid, is synthesized in the transsulfuration pathway from homocysteine and serine and is required for glutathione synthesis. Glutathione plays a potential role in the etiology of multiple diseases including cancer, because it is an important antioxidant that mitigates oxidative stress (42,43). In our analyses, cysteine also correlated with plasma PLP. This correlation was not surprising because PLP is a coenzyme for both cystathionine β-synthase and cystathionine γ-lyase, which are essential for the de novo synthesis of cysteine (44).

A key strength of this study is the large sample size. In addition, we investigated a fairly comprehensive set of nutritional and one-carbon biomarkers, and for the first time in this context the inflammatory marker SAA. Because of the design of the WHI-OS, we were able to investigate trends in associations across the various stages of folic acid fortification. A limitation may be the lack of generalizability of the results presented; the work is representative for women but not for men or the population at large. Potentially, some of the women in the study had preclinical disease at the time of recruitment, and biomarker measurements were only available at 1 time point. Additionally, indicators of kidney function were not assessed, which can be determinants of homocysteine and cysteine metabolism in women. Furthermore, the cross-sectional design of the study is a limitation; reverse causation cannot be ruled out.

TABLE 4 Linear regression models predicting inflammatory biomarkers in postmenopausal women, adjusted for age and BMI[1]

| | Serum CRP (mg/L) | | | | SAA (mg/L) | | | |
| | Unadjusted | | Adjusted | | Unadjusted | | Adjusted | |
	β	P value	β	P value	β	P value	β	P value
Nutritional biomarker								
Plasma PLP (nmol/L)	−1.47	<0.0001	−1.46	<0.0001	−0.91	<0.0001	−1.04	<0.0001
Plasma vitamin B-12 (pg/mL)	0.05	0.49	0.10	0.19	0.14	0.04	0.23	0.002
RBC folate (μg/L)	0.25	0.0004	0.32	<0.0001	0.17	0.02	0.25	0.001
Plasma folate (μg/L)	1.44	0.28	2.50	0.08	0.46	0.72	1.64	0.25
Integrated marker of one-carbon status								
Plasma homocysteine (μmol/L)	14.32	0.02	21.04	0.002	16.81	0.004	26.61	<0.0001
Plasma cysteine (μmol/L)	−2.19	<0.0001	−2.07	<0.0001	−0.70	0.14	−0.87	0.08

[1] $n = 1639$. CRP, C-reactive protein; PLP, pyridoxal-5′-phosphate; SAA, serum amyloid A.

In conclusion, biomarkers of inflammation were associated with the nutritional markers plasma PLP and RBC folate as well as with integrated indicators of one-carbon status (homocysteine and cysteine). These results suggest that the identification of biomarker patterns may provide a step forward in understanding links between inflammatory processes, nutritional status, and certain diseases characterized by chronic inflammatory reactions, such as cancer. Mechanistic studies analyzing the pathways behind the presented correlations are needed to further describe mechanisms and to establish causal relations.

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