

Association of plasma B-6 vitamers with systemic markers of inflammation before and after pyridoxine treatment in patients with stable angina pectoris^{1–3}

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ABSTRACT

Background: A negative association between systemic markers of inflammation and plasma vitamin B-6 has been observed in population-based and patient cohorts; however, vitamin B-6 (pyridoxine) treatment has mostly failed to improve inflammatory indexes.

Objective: We aimed to assess the effect of pyridoxine treatment on B-6 vitamer and inflammatory marker relations.

Design: We measured pyridoxal 5'-phosphate (PLP), pyridoxal, 4-pyridoxic acid (PA), C-reactive protein (CRP), neopterin, and the kynurenine-to-tryptophan ratio (KTR) in plasma and the white blood cell count (WBC). A partial Spearman's correlation was used to assess associations of B-6 vitamers with inflammatory markers before and after daily treatment with 40 mg pyridoxine hydrochloride. Generalized additive models and segmented regression analysis were used for nonlinear relations.

Results: A 9–60-fold increase in B-6 vitamer concentrations over baseline values was observed after 28 d of treatment with pyridoxine. PLP was negatively associated with all 4 inflammatory markers at baseline and, predominantly, with CRP and KTR at day 28. The catabolite PA was positively associated with neopterin and KTR before and after treatment. The dose-response relation between CRP and B-6 vitamers at day 28 was nonlinear, with an increased steepness of slope at CRP >7 mg/L. Finally, changes in B-6 vitamer concentrations were correlated with changes in inflammatory marker concentrations over a time span of 4 wk.

Conclusions: The associations between plasma vitamin B-6 and inflammatory markers were preserved or even increased after pyridoxine treatment. The results suggest that the acute phase and activated cellular immunity are associated with increased cellular uptake and catabolism of vitamin B-6, respectively. *Am J Clin Nutr* 2012;95:1072–8.

INTRODUCTION

Vitamin B-6 mainly exists in plasma in the following 3 forms: pyridoxal 5'-phosphate (PLP⁴; active coenzyme), pyridoxal (PL), and 4-pyridoxic acid (PA; catabolite). PLP is a cofactor in >100 enzymatic reactions in the human body, including the metabolism of amino acids, neurotransmitters, nucleic acids, and lipids. PLP is also important in energy homeostasis, mainly in the degradation of glycogen and in gluconeogenesis (1). The plasma concentration of PLP is regarded as the most relevant marker of vitamin B-6 status (2).

In cross-sectional studies, low plasma concentrations of PLP have been observed in various diseases, including coronary artery

disease (3–5), HIV infection (6), cancer (7), rheumatoid arthritis (8, 9), and in individuals with elevated markers of inflammation in population-based and patient cohorts (10, 11). Thus, inflammation seems to be a common link between low vitamin B-6 status and a diversity of clinical conditions. It has been speculated that these associations reflect an increased requirement for vitamin B-6 (10, 12). Experimental results indicated that PLP availability may modify both cellular and humoral immune responses (13, 14). However, clinical trials with vitamin B-6 intervention in patients with chronic inflammatory conditions have, in most cases, given negative results with regard both to the clinical expression of disease and to concentrations of circulating inflammatory markers (15–18).

Serum concentrations of the acute-phase reactant C-reactive protein (CRP) is the most frequently used marker for (acute-phase) immune activation, and with the advent of high-sensitivity CRP, chronic low-level inflammation may also be captured by this index (19). Also, the total white blood cell count (WBC) often increases during the acute-phase response.

The cytokine interferon- γ (IFN- γ) is released from antigen-stimulated T-helper 1 (Th1) cells and is a hallmark of activated cellular immunity. IFN- γ activates GTP cyclohydrolase I, which produces neopterin in monocytes and macrophages and the ubiquitous enzyme indoleamine (2,3)-dioxygenase that converts tryptophan to kynurenine. Thus, plasma neopterin and the kynurenine-to-tryptophan ratio (KTR) are useful markers of IFN- γ -activated Th1 immune responses (20).

We investigated the association between plasma concentrations of B-6 vitamers PLP, PL, and PA and the inflammatory

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⁴ Abbreviations used: CRP, C-reactive protein; GAM, generalized additive model; IFN- γ , interferon- γ ; KTR, kynurenine-to-tryptophan ratio; PA, pyridoxic acid; PL, pyridoxal; PLP, pyridoxal 5'-phosphate; Th1, T-helper 1; WBC, white blood cell count.

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markers CRP, WBC, KTR, and neopterin in patients with stable angina pectoris before and after treatment with pyridoxine for 28 d. The aim of the study was to assess if such treatment affected associations between B-6 vitamers and inflammatory indexes. Data were derived from the Western Norway B-Vitamin Intervention Trial (www.clinicaltrials.gov; NCT00354081) that included a total of 3090 patients who were undergoing angiography for suspected coronary artery disease.

SUBJECTS AND METHODS

Study participants

Participants in the Western Norway B-Vitamin Intervention Trial were recruited during the period 2000–2004 at 2 university hospitals in Western Norway. The study protocol, selection criteria, and patient characteristics at baseline have been described previously (18). Patients ($n = 3090$) were randomly allocated to the following 4 intervention groups: 1) 40 mg pyridoxine hydrochloride + 0.8 mg folic acid + 0.4 mg B-12, 2) 0.8 mg folic acid + 0.4 mg B-12, 3) 40 mg pyridoxine hydrochloride, and 4) placebo. All participants were instructed to take the study medication in the morning. We excluded patients with acute coronary syndrome at admission ($n = 461$), patients who reported consumption of B vitamin supplements before inclusion ($n = 360$), and participants who discontinued their study medication during the first 28 d of the intervention ($n = 159$). Thus, in the 2 groups who received vitamin B-6 treatment, a total of 1088 patients with stable coronary artery disease were eligible for analyses, and in the 2 non-vitamin B-6 treatment groups, 1099 patients were eligible.

Clinical data and laboratory analyses

Clinical information and blood samples were obtained at baseline, at a follow-up visit 28 d after random assignment, after 1 y, and at a final study visit after 3 y. At baseline, nurses or physicians interviewed patients by using trial-specific questionnaires. The smoking status of subjects was assessed by asking participants if they were current or former smokers and, for former smokers, how much time had passed since they had quit smoking. Vitamin supplementation was assessed by asking about regular use of over-the-counter vitamin supplements. Determinations of plasma B-6 vitamers, neopterin, kynurenine, tryptophan, and creatinine were performed at the laboratory of Bevitall AS by using assays that were based on liquid chromatography–tandem mass spectrometry or gas chromatography–mass spectrometry (21). CRP was determined in serum with the Behring nephelometer II system (Latex CRP mono; Behring Diagnostics), and the WBC was determined in EDTA-blood with hematology analyzers: Abbott Cell Dyn 4000 (Abbott Diagnostics) or ADVIA 120 (Bayer Diagnostics). Blood samples were collected under nonfasting conditions, and samples were stored at -80°C for an average of 5.6 y before analysis. With data from 117 plasma samples measured on 2 occasions, we determined that neopterin, kynurenine, tryptophan, PLP, PL, and PA were stable during storage for 6 y.

Statistical methods

The distributions of all plasma metabolites were right skewed, and therefore, central values are reported as medians. In regression analyses, all continuous variables were log transformed,

and results are presented after back-transformation. The effect of vitamin B-6 treatment was analyzed by comparing the change (from days 0 to 28 within subjects) for the 2 groups (vitamin B-6 compared with non-vitamin B-6 treatment) by using a 2-sample t test with unequal variances. Because of the bimodal shapes of PL and PA distributions at day 28, we used a partial Spearman's correlation (with adjustment for covariates) for the evaluation of associations of B-6 vitamers with inflammatory markers before and after pyridoxine treatment. Generalized additive model (GAM) regression was used to obtain dose-response curves for B-6 vitamers versus inflammatory markers, and segmented regression was used to find the breakpoint between linear segments (with assumption of a 2-segmented model) by using starting values on the basis of a visual inspection of GAM curves. Unadjusted Spearman's correlation and GAM were used for evaluating the association between average changes in B-6 vitamers over a time span of 28 d with corresponding changes in inflammatory markers. Change was defined as

$$\begin{aligned} \text{Log}(\text{value at day 28}) - \text{log}(\text{value at baseline}) \\ = \text{log}(\text{day 28} \div \text{baseline}) \end{aligned} \quad (1)$$

In graphical presentations, values were back transformed and reported as the fold difference. Effect modification was evaluated by including interaction (product) terms in regression models. All correlations, when specified in the text, were significant at $P < 0.05$. R version 2.12.1 software (22) was used for all statistical analyses with the “mgcv” package used for GAM analysis and the “segmented” package used for segmented regression.

RESULTS

The median (5th–95th percentile) age of the study population was 61.6 y (45.3–77.0 y), and 81.4% of the study population were men. The frequency of self-reported current smokers was 22.8%. From baseline to day 28, median PLP, PL, and PA concentrations in vitamin B-6 treatment groups increased ~ 9 -, ~ 60 -, and ~ 40 -fold, respectively. Increases were larger for women than for men ($P < 0.02$). Plasma concentrations of B-6 vitamers, inflammatory markers, and other metabolites at the 2 time points are shown in **Table 1**. Except for WBC, all indicators of inflammation were higher at day 28. However, none of the inflammatory markers were affected by vitamin B-6 treatment ($P > 0.05$ for all).

B-6 vitamers at baseline and day 28

Cumulative distribution curves for the 3 vitamers PLP, PL, and PA at baseline and day 28 are shown in **Figure 1**. Notably, at day 28, distributions of PL and PA were bimodal with maxima at ~ 85 and ~ 900 nmol/L for PL, and ~ 180 and ~ 1340 nmol/L for PA (as estimated from distribution curves, not shown). The Spearman's correlation coefficient between PL and PA increased from 0.57 at baseline to 0.94 at day 28, whereas correlations between PLP and PL decreased from 0.68 to 0.39, and between PLP and PA, correlations decreased from 0.42 to 0.36.

B-6 vitamers and inflammatory markers

Adjusted Spearman's correlations between B-6 vitamers and the 4 inflammatory markers at baseline and day 28 are shown in

TABLE 1

Plasma concentrations of B-6 vitamers, inflammation indexes, and metabolites at baseline and day 28¹

	Non-vitamin B-6 treatment groups (n = 1099)			Vitamin B-6 treatment groups (n = 1088)			Effect of vitamin B-6 treatment	
	Baseline	Day 28	P ³	Baseline	Day 28	P ³	r ²	P ⁴
PLP (nmol/L)	38.5 (18.8–83.9)	39.2 (18.9–84.7)	0.13	39.1 (17.6–91.3)	355 (128–575)	<0.01	0.74	<0.01
PL (nmol/L)	8.90 (4.97–16.7)	9.08 (5.25–18.1)	0.04	9.14 (5.03–18.4)	545 (33.5–1507)	<0.01	0.56	<0.01
PA (nmol/L)	23.3 (14.2–44.5)	24.6 (15.6–48.6)	<0.01	23.7 (13.8–51.0)	926 (86.5–2011)	<0.01	0.55	<0.01
CRP (mg/L)	1.82 (0.35–10.5)	1.94 (0.33–27.5)	<0.01	1.64 (0.31–13.7)	1.86 (0.31–25.1)	<0.01	0.58	0.90
WBC (×10 ⁶ /mL)	6.9 (4.5–10.7)	6.7 (4.4–10.6)	<0.01	6.9 (4.5–10.6)	6.9 (4.4–10.2)	0.03	0.68	0.64
KTR (nmol/μmol)	23.5 (15.9–38.0)	25.0 (16.8–42.4)	<0.01	23.8 (15.9–40.1)	25.6 (16.5–42.7)	<0.01	0.71	0.46
Neopterin (nmol/L)	7.78 (5.17–13.8)	8.26 (5.34–16.0)	<0.01	7.70 (5.08–14.4)	8.24 (5.16–15.9)	<0.01	0.68	0.40
Creatinine (mmol/L)	73.7 (53.5–100)	74.9 (54.8–102)	<0.01	73.3 (52.9–102)	75.4 (55.5–105)	<0.01	0.80	0.56

¹ All values are medians; 5th–95th percentiles in parentheses. CRP, C-reactive protein; KTR, kynurenine-to-tryptophan ratio; PA, pyridoxic acid; PL, pyridoxal; PLP, pyridoxal 5'-phosphate; WBC, white blood cell count.

² Spearman's correlation between baseline and day 28 for pairwise observations ($P < 0.01$ for all). Values for B-6 vitamers were based on non-vitamin B-6 treatment groups.

³ Mann-Whitney U test for pairwise observations.

⁴ Change (from days 0 to 28 within subjects) was compared for the 2 groups (vitamin B-6 compared with non-vitamin B-6 treatment) by a 2-sample t test with unequal variances.

Figure 2. In model 1, adjustments included age, sex, and study center. In model 2, we adjusted, in addition, for current smoking, creatinine, and each of the other inflammation indexes. At baseline, PLP was negatively associated with all 4 markers, whereas PL was negatively associated with CRP and WBC only. In contrast, PA was positively associated with KTR and neopterin. The pattern of correlations at day 28 was similar. The main differences were a stronger association of PLP with CRP and KTR, and PA became inversely associated with CRP. Although this study focused primarily on associations at baseline and day 28, analyses were also done at day 365. Most findings were similar at this time point (see Figure S1 under “Supplemental data” in the online issue for results).

B-6 vitamer–CRP relation

The dose-response relation between B-6 vitamers and CRP by using GAM regression is shown in **Figure 3**. For PL and PA in particular, the inverse association with CRP at day 28 was

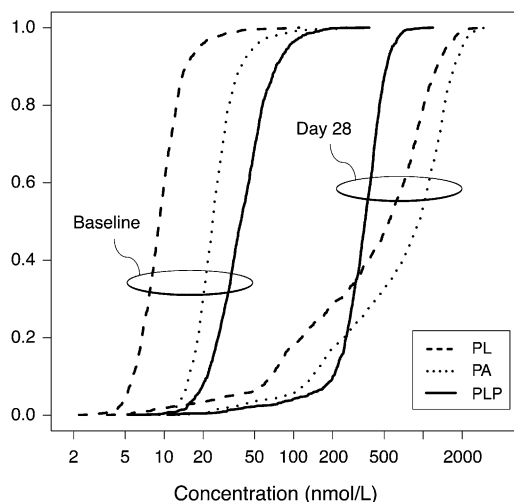


FIGURE 1. Cumulative distribution curves for plasma B-6 vitamers before and after 28 d of daily oral treatment with 40 mg pyridoxine-hydrochloride. PA, pyridoxic acid; PL, pyridoxal; PLP, pyridoxal 5'-phosphate.

markedly nonlinear with a change in slope and increased negative association at higher CRP concentrations. Significant breakpoints in the range 6.9–7.1 mg/L for biphasic relations between CRP and PL, PA, and PLP at day 28 and between CRP and PL at baseline were shown by using segmented regression. Nonlinear associations for other B-6 vitamer and inflammatory marker combinations were generally less pronounced, and nonlinear associations of PL and PA with KTR or neopterin were absent (results not shown).

Changes in B-6 vitamer concentrations compared with changes in inflammation status from baseline to day 28

Concentrations of inflammatory markers varied between baseline and day 28 as reflected by intraindividual correlations that ranged from 0.58 for CRP to 0.71 for KTR (Table 1). Therefore, we analyzed changes in concentrations from baseline to day 28 for all B-6 vitamers as a function of the corresponding change in each inflammatory marker. In non-vitamin B-6 treatment groups, Δ PLP was negatively correlated with Δ CRP and Δ KTR (Spearman's correlation coefficients of -0.13 and -0.12 , respectively), and Δ PL was negatively correlated with Δ CRP ($r = -0.09$). In contrast, Δ PA was positively correlated with Δ KTR and Δ neopterin ($r = 0.13$ and 0.15 , respectively). In the vitamin B-6 intervention groups, changes in all B-6 vitamers were negatively correlated with Δ CRP with r ranging from -0.20 to -0.07 , and Δ PLP was also correlated to Δ KTR ($r = -0.15$). For changes in the other vitamin B-6 species and inflammatory marker combinations, associations were weaker or nonsignificant. Graphical representations of correlations between Δ PLP and Δ CRP as modeled by GAM regression are shown in **Figure 4**. Notably, as shown in Figure 4, reduced CRP over a time span of 28 d was associated with higher PLP in both vitamin B-6 nontreatment and treatment groups. Similarly, reduced neopterin and KTR were associated with lower PA in nontreatment groups (results not shown).

Sensitivity analysis

A large percentage of low values of PL and PA at day 28 (Figure 1) was shown in a subgroup of participants (24%) who



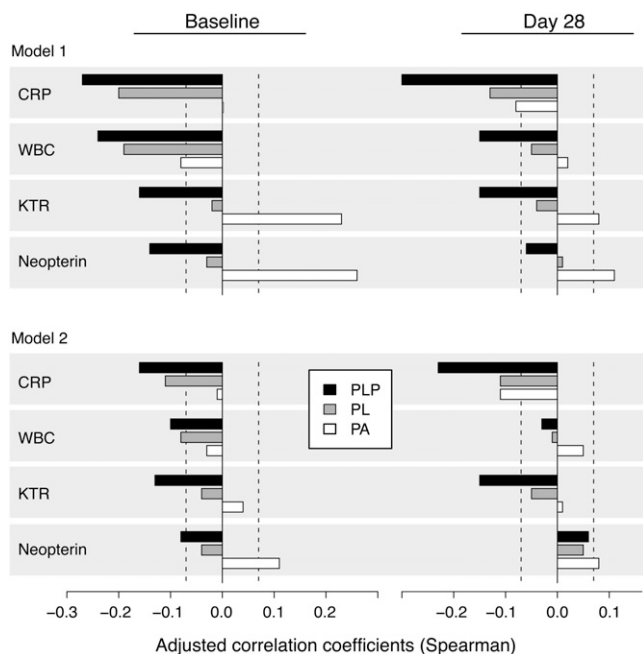


FIGURE 2. Adjusted (partial) correlations of plasma B-6 vitamers with inflammatory markers at baseline and day 28. Model 1 included adjustment for age, sex, and study center, and model 2 included additional adjustment for current smoking, creatinine, and other inflammation markers. Correlation coefficients >0.07 (dashed lines) were significant at $P < 0.05$. CRP, C-reactive protein; KTR, kynurenine-to-tryptophan ratio; PA, pyridoxic acid; PL, pyridoxal; PLP, pyridoxal 5'-phosphate; WBC, white blood cell count.

had their blood sample drawn at a local physician's offices. After the removal of this group, we showed that the correlations between B-6 vitamers and CRP were slightly stronger. For PLP and PL, the differences were significant (P -interaction = 0.02 and 0.04, respectively). See Table S1 under "Supplemental data" in the online issue for the results of this analysis.

DISCUSSION

Principal findings

We assessed the relation between plasma concentrations of B-6 vitamers and 4 inflammatory markers (ie, CRP, WBC, KTR, and neopterin) before and after pyridoxine treatment in a population with stable angina pectoris. The intervention caused a 9–60-fold increase in concentration of B-6 vitamers over baseline values. PLP was negatively associated with all 4 inflammatory markers at baseline and with CRP and KTR at day 28. PA, in contrast, was positively associated with KTR and neopterin at both time points. At day 28, all B-6 vitamers were negatively associated with CRP, and dose responses were nonlinear with markedly steeper slopes at $\text{CRP} > 7 \text{ mg/L}$. Finally, in vitamin B-6 and non-vitamin B-6 intervention groups, we showed that changes in inflammatory marker concentrations over the time span of 28 d were correlated with changes in B-6 vitamers concentrations in line with the cross-sectional results.

B-6 vitamers after pyridoxine treatment

Baseline vitamin B-6 concentrations were comparable to previously reported concentrations in healthy subjects (21, 23, 24). The

vitamin B-6 intervention was given as pyridoxine hydrochloride. After digestion, pyridoxine is metabolized in the liver to pyridoxine 5'-phosphate and then to PLP. Pharmacokinetic studies have shown that the plasma pyridoxine concentration rises immediately after administration and then disappears after a few hours and is shortly followed by a transient, almost parallel, increase and decrease in PL and PA during the next 5–10 h. In contrast, the increase in PLP is moderate but more persistent (25, 26). These findings imply that most of the newly formed PLP is converted to PL, of which some, along with PA, is released into plasma, where it is detected for several hours until most is catabolized in the liver and kidneys, whereas a comparatively minor portion is retained in the liver as PLP or exported to plasma as albumin-bound PLP.

The low peak of bimodal distributions of PL and PA could be explained by blood sampling at the end of the workday, and, thus, 7–9 h after the study medication was taken, and was shown mostly for the subset of participants (24%) who had their blood drawn by their local physician.

B-6 vitamers and acute-phase markers

A notable finding of the current study was the strong association of PLP, PL, and PA with CRP after 1 mo of treatment with high doses of vitamin B-6 (ie, 15–20 times the Recommended Dietary Allowance) (27). Clues to the mechanism behind these relations may be in the PL and PA associations. Because PL is the form taken up by cells, the considerable reductions in both PL and PA at $\text{CRP} > 7 \text{ mg/L}$ indicated that PL was removed from the plasma, presumably, by increased cellular uptake into peripheral tissue. The common CRP threshold value for all vitamers of $\sim 7 \text{ mg/L}$ indicated that this may be a prime mechanism only or mainly at the full-blown acute phase. At baseline, PL concentrations were low and probably closer to a steady state relation with plasma PLP.

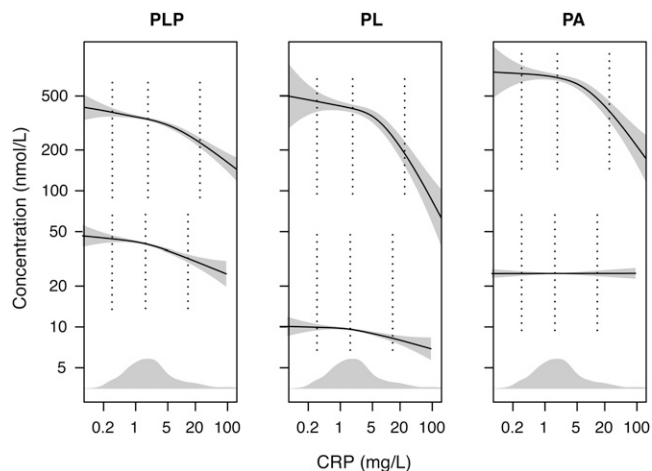


FIGURE 3. Dose-response curves with 95% CI (shaded areas) for the relation between B-6 vitamers and CRP at baseline (lower curves) and day 28 (upper curves) by generalized additive model regression. Analyses were adjusted for age, sex, study center, creatinine, and current smoking. A density plot for the CRP distribution at day 28 is included. Dotted lines indicate the 5th, 50th, and 95th percentiles of CRP at baseline (lower lines) and day 28 (upper lines). Analyses were done on log-transformed values, and values are shown after back-transformation. CRP, C-reactive protein; PA, pyridoxic acid; PL, pyridoxal; PLP, pyridoxal 5'-phosphate.

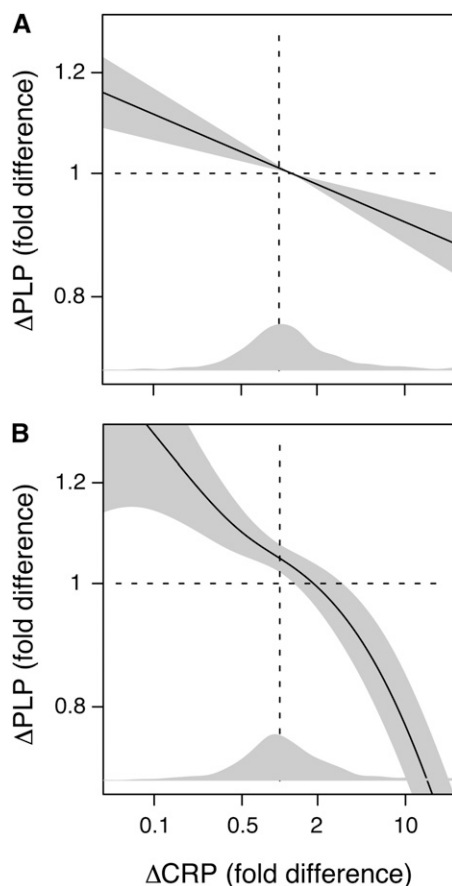


FIGURE 4. Dose-response relation with 95% CIs (shaded area) between Δ PLP and Δ CRP over a time span of 28 d by using unadjusted generalized additive regression. A: Vitamin B-6 nonintervention groups. B: Vitamin B-6 intervention groups. Density plots for the distribution of Δ CRP values are included. Dashed lines indicate a ratio of 1, or no change, in each dimension. Analyses were done on log-transformed values, and values are shown after back-transformation. CRP, C-reactive protein; PLP, pyridoxal 5'-phosphate.

Still, curved (convex) relations with CRP were also shown for PLP and PL at that time point.

B-6 vitamers and cellular immunity markers

PLP was negatively associated with KTR after adjustments for CRP and other inflammatory markers at baseline, and similar to CRP, the association was not weakened after 1 mo of high vitamin B-6 intake and markedly higher PLP amounts. Again, clues to possible causal mechanisms may be in the associations of inflammatory markers with PL and PA: Unlike the acute-phase markers, the cellular immunity markers were not associated with PL, but both markers were positively associated with PA. These findings suggested that increased catabolism of vitamin B-6 rather than increased cellular uptake is a likely mechanism behind the inverse PLP-Th1 marker associations.

Longitudinal findings

A third important finding in this study was the correlation of changes in concentrations of B-6 vitamers with changes in in-

flammatory marker concentrations within the time frame of 28 d. First, these findings are in line with and confirm the cross-sectional results, and second, the findings indicate a dynamic relation between plasma concentrations of B-6 vitamers and inflammatory markers over a relatively short time span. The transiency of low plasma PLP has been reported in a previous study in which plasma PLP decreased by 45% during the acute phase after myocardial infarction but returned to normal before discharge from the hospital (3). In the current study, marked responses in concentrations of B-6 vitamer were detected despite the relatively moderate changes in inflammatory status that occurred in our study population.

Vitamin B-6 demand, tissue uptake, and acute-phase response

An increased cellular demand for PLP and, therefore, uptake of vitamin B-6 are likely consequences of quantitative changes in protein turnover that accompany the acute-phase reaction (28, 29). One of the key signaling molecules in these processes is IL-6, which is a coactivator of the hypothalamic-pituitary-adrenalin axis (30). An elevation of cortisol and other stress hormones elicits protein degradation in muscle, gut, and connective tissue, supplying amino acids for the synthesis of immunomodulating proteins, immune cell proliferation, cell repair at the site of the lesion, and energy production through gluconeogenesis. A direct link between IL-6, CRP, and plasma vitamin B-6 concentrations is implied by IL-6 being a major inducer of CRP but also an enhancer of the tissue-nonspecific alkaline phosphatase activity that is necessary for cellular vitamin B-6 uptake (31, 32). In a rat study, redistribution between tissues was demonstrated by low PLP in plasma and liver accompanied by unaffected erythrocyte PLP during inflammation (15).

Oxidative stress, vitamin B-6 catabolism, degradation, and cellular immunity

Strong associations have been shown between neopterin formation and the release of reactive oxygen species from macrophages (33). Oxidative stress is accompanied by the generation of endogenous cytotoxic aldehydes derived from the metabolism of lipids and sugars, which normally is counteracted by upregulation of ubiquitous NAD⁺-dependent aldehyde dehydrogenases (34). In rats, this enzyme was shown to metabolize PL to PA in a number of tissues (35) and could possibly explain the positive association of PA with neopterin and KTR; however, Merrill et al (36) did not find this enzyme activity in the human liver. Several vitamin B-6 species have been shown to have antioxidant properties (37, 38). Therefore, nonspecific oxidative degradation as well as increased catabolism may contribute to the low PLP associated with these markers.

Strength and limitations

Strengths of the study included its large size and longitudinal aspects of the study design. The self-reported adherence to study medication was 94.9% during the first 28 d of intervention, and the pretrial use of vitamin B-6 supplements was low (11.7%). Bimodal distributions of PL and PA posed an analytic challenge; however,

sensitivity analyses showed that the strong associations of B-6 vitamers with CRP were maintained after the exclusion of participants with low PL and PA at day 28. Our findings were obtained in a cohort with mild inflammation related to cardiovascular disease, and the results may not necessarily be applicable to healthy populations or to patients with severe chronic inflammatory diseases.

In conclusion, we showed that negative associations of B-6 vitamers with several markers of inflammation were mostly preserved and, for some, even strengthened after 28 d of pyridoxine intervention. Notable characteristics and differences in the vitamin B-6–inflammatory marker associations indicated that the acute-phase response is associated with increased cellular uptake of vitamin B-6, whereas accelerated catabolism and oxidative degradation may be more important during activated cellular immunity. The longitudinal findings suggest that altered disposition and metabolism of vitamin B-6 are adaptive processes that respond over short time intervals (days or weeks) to relatively moderate changes in inflammatory status.

The authors' responsibilities were as follows—AU, ON, and PMU: study concept and design; ERP and ØM: acquisition of the data; AU: analysis of the data; AU, PMU, and ERP: drafting of the manuscript; AU, PMU, ERP, ØM, and ON: critical revision of the manuscript for important intellectual content; and AU: primary responsibility for the final content of the manuscript. All authors read and approved the final manuscript. None of the authors had a conflict of interest.

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