

Substrate product ratios of enzymes in the kynurenine pathway measured in plasma as indicators of functional vitamin B-6 status^{1–3}

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ABSTRACT

Background: Tryptophan metabolism through the kynurenine pathway includes 2 vitamin B-6 [pyridoxal 5'-phosphate (PLP)]-dependent enzymes. We recently showed that plasma 3-hydroxykynurenine (HK) was elevated at low PLP concentrations.

Objective: We further evaluated and characterized kynurenine-based indexes as possible markers of functional B-vitamin status in plasma.

Design: Cross-sectional and longitudinal data were derived from the Western Norway B-vitamin Intervention Trial, including PLP, kynurenine, HK, kynurenic acid (KA), anthranilic acid, xanthurenic acid (XA), and 3-hydroxyanthranilic acid (HAA) measured in plasma at 2 time points. Partial Spearman's correlation, generalized additive models, and receiver operating characteristic (ROC) analysis were used to assess associations of kynurenines with PLP.

Results: Ratios HK:XA, HK:HAA, and HK:KA showed markedly stronger negative correlations with PLP than did HK alone (Spearman's $\rho = -0.36, -0.29, \text{ and } -0.31$ compared with -0.18 , respectively). All associations were nonlinear, with the strongest relation at low PLP. In the ROC analysis, areas under the curve for discriminating low PLP (less than the fifth percentile; 18.6 nmol/L) were 0.78, 0.78, and 0.74, respectively, compared with 0.65 for HK. Oral treatment with 40 mg pyridoxin hydrochloride for 28 d reduced the ratios by up to 60%, with strongest reductions for subjects with low plasma PLP at baseline. Whereas HK was associated with kidney function and several inflammatory markers, such associations were abolished or attenuated for the ratios.

Conclusion: Plasma values of HK:XA and HK:HAA, which are substrate-product pairs for kynurenine transaminase and kynureninase, respectively, may reflect the intracellular availability of the cofactor (PLP) and, therefore, present as potential markers of functional vitamin B-6 status. *Am J Clin Nutr* 2013;98:934–40.

INTRODUCTION

The first step in tryptophan catabolism, which forms kynurenine, is catalyzed by the hepatic tryptophan 2,3-dioxygenase or the ubiquitous indoleamine 2,3-dioxygenase (IDO)⁴ (1). The latter is induced by inflammatory stimuli, most importantly interferon- γ . The kynurenine:tryptophan ratio (KTR) in plasma is considered a specific marker of IDO activity and is highly correlated to neopterin, which is a macrophage-derived metabolite that increases after interferon- γ stimulation (2). The subsequent steps in the kynurenine pathway involve the following

2 vitamin B-6 [pyridoxal 5'-phosphate (PLP)]-dependent enzymes: kynureninase and kynurenine transaminase (KAT). Kynureninase lies on the main pathway toward acetyl-CoA or NAD synthesis and converts kynurenine to anthranilic acid (AA) and 3-hydroxykynurenine (HK) to 3-hydroxyanthranilic acid (HAA). KAT converts the same 2 substrates into kynurenic acid (KA) and xanthurenic acid (XA), respectively (**Figure 1**).

One of the first discovered metabolic consequences of PLP deficiency (in rats) was the increased excretion of XA in urine after a tryptophan load (3). Subsequently, increased excretion of a number of kynurenines, including HK, were shown in vitamin B-6-deficient humans (4, 5). In a case report, the HK:HAA ratio was proposed as the most sensitive and specific indicator of increased PLP dependency (6). HK:HAA was subsequently used, with or without a tryptophan load, to determine vitamin B-6 status in patients (7–10). However, the method was criticized for not being strictly specific to vitamin B-6 status (10).

Interest in vitamin B-6 status has come from repeated observations of low concentrations of vitamin B-6 indexes in

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⁴ Abbreviations used: AA, anthranilic acid; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; GAM, generalized additive model; HAA, 3-hydroxyanthranilic acid; HK, 3-hydroxykynurenine; IDO, indoleamine 2,3-dioxygenase; KA, kynurenic acid; KAT, kynurenine transaminase; KTR, kynurenine:tryptophan ratio; PLP, pyridoxal 5'-phosphate; ROC, receiver operating characteristic; WENBIT, Western Norway B-vitamin Intervention Trial; XA, xanthurenic acid.

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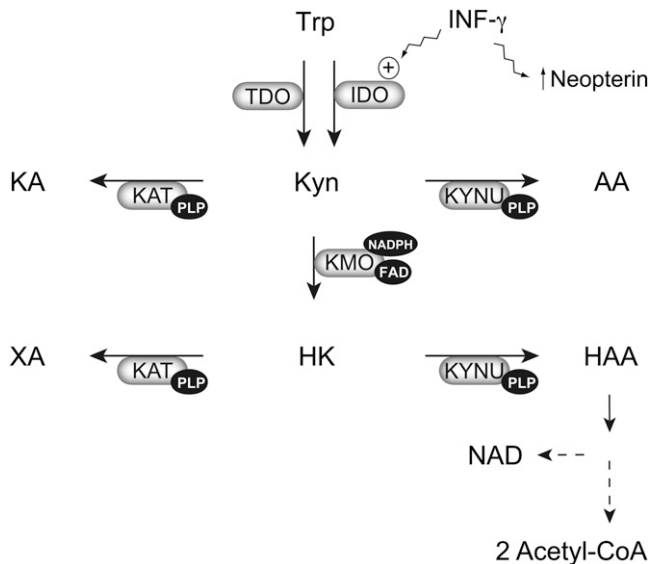


FIGURE 1. Trp metabolism through the kynurenine pathway. Enzymes and cofactors are depicted. IDO is activated by inflammatory stimuli including INF- γ , which also stimulates macrophages to produce neopterin. TDO is activated by Trp and glucocorticoids. The immediate product of TDO and IDO (ie, formylkynurenine) is not shown. AA, anthranilic acid; HAA, 3-hydroxyanthranilic acid; HK, 3-hydroxykynurenine; IDO, indoleamine 2,3-dioxygenase; INF- γ , interferon- γ ; KA, kynurenic acid; KAT, kynurenine transaminase; KMO, kynurenine monooxygenase; Kyn, kynurenine; KYNU, kynureninase; PLP, pyridoxal 5'-phosphate; TDO, tryptophan 2,3-dioxygenase; Trp, tryptophan; XA, xanthurenic acid.

diseases associated with chronic or acute inflammation (11, 12). This appears to be part of a more general phenomenon because circulating concentrations of many vitamins and micronutrients are reduced during inflammatory conditions (13). One of the mechanisms proposed is the redistribution of vitamins and their binding proteins into intercellular space because of increased vascular permeability or active uptake into tissues (13–15). Consequently, low plasma concentrations may not necessarily indicate intracellular vitamin B-6 deficiency. To resolve such questions, sensitive functional and metabolic indicators are needed.

We recently developed a liquid chromatography–tandem mass spectrometry–based assay for the sensitive quantification of tryptophan and the 6 first metabolites in the kynurenine pathway in plasma (16) (Figure 1). With the use of this assay, we showed that plasma HK was elevated at PLP concentrations <20 nmol/L (17), which is a cutoff that is commonly associated with vitamin B-6 deficiency. In the current study, we examined HK:HAA, which is a substrate product ratio of kynureninase, the corresponding substrate product of KAT, HK:XA, and other kynurenine-based indexes as potential markers of vitamin B-6 status.

SUBJECTS AND METHODS

Subjects

The study included 2628 adults ($>98\%$ whites), of the Western Norway B-Vitamin Intervention Trial (WENBIT; www.clinicaltrials.gov; NCT00354081) who were undergoing a coronary angiography for suspected coronary artery disease between 1999 and 2004 at the Haukeland University Hospital (Bergen, Norway) and Stavanger University Hospital (Stavanger, Norway).

Participants ($n = 460$) classified as having acute coronary syndrome were excluded from the current study. Of the included subjects, 2584 participants had stable angina pectoris, and 44 subjects had aortic stenosis. Details of the WENBIT study have been published elsewhere (18). In the current study, we used data at baseline and after 28 d of follow-up for participants randomly allocated to 4 treatment groups in a 2×2 factorial design. The 4 treatment groups consisted of 1) vitamin B-6 (40 mg pyridoxine hydrochloride), folic acid (0.8 mg), and vitamin B-12 (0.4 mg); 2) folic acid and vitamin B-12; 3) vitamin B-6; and 4) a placebo.

Written informed consent was obtained from all participants. The study protocol was in accordance with the principles of the Declaration of Helsinki, and the trial was approved by the Regional Committee for Medical and Health Research Ethics, the Norwegian Medicines Agency, and the Data Inspectorate.

Clinical data and laboratory analyses

Nurses or physicians interviewed patients at baseline by using trial-specific questionnaires. Smoking status was assessed by asking participants whether they were current or former smokers and, if they were former smokers, how long it had been since they quit smoking. Vitamin supplementation was assessed by asking about the regular use of over-the-counter vitamin supplements. Blood (plasma) samples obtained at baseline and after 28 d of vitamin treatment were stored at -80°C for a mean duration of 5.6 y before analysis. Plasma concentrations of PLP, riboflavin, tryptophan, kynurenine, KA, AA, HK, XA, HAA, neopterin, and creatinine were measured by liquid chromatography–tandem mass spectrometry (16, 19). Concentrations of kynurenines were similar to previous measurements in fresh samples (20). The KTR was calculated by dividing the plasma concentration of kynurenine (in nmol/L) by the concentration of tryptophan (in $\mu\text{mol/L}$). The estimated glomerular filtration rate (eGFR) per 1.73 m 2 was calculated on the basis of the Chronic Kidney Disease Epidemiology Collaboration formula (21). C-reactive protein (CRP) was determined in serum by an ultrasensitive immunoassay applying the Behring nephelometer II system (Latex CRP mono; Behring Diagnostics).

Statistical methods

Associations between variables were assessed with partial Spearman's correlation using simple and extended models. When the whole cohort ($n = 2628$) was analyzed, correlation coefficients >0.06 were significant at $P < 0.01$. Interaction was evaluated by the inclusion of product terms in multiple linear regression models. Nonlinear associations were assessed by using generalized additive models (GAMs) adjusted for age, sex, and center. The effect of treatment with vitamin B-6 was evaluated in statistical models by using the metabolite or ratio at 1 mo divided by the metabolite or ratio at baseline (treatment ratio) as the outcome. Taking advantage of the 2×2 factorial design, we regressed each treatment ratio on the factors vitamin B-6 treatment, folic acid+vitamin B-12 treatment, and their product term. Independence between treatment arms, which was defined as the nonsignificance ($P > 0.05$) of the product term, was found for all metabolites and ratios analyzed. In addition, GAMs were used to evaluate treatment effects as a function of

baseline PLP with adjustment for treatment group. In parametric analyses (multiple linear regression and GAMs), all continuous variables, except age, were log-transformed. Missing data were handled by listwise deletion. All analyses were performed using R for Macintosh software (version 2.15.2; The R-Foundation for Statistical Computing) by using the packages mgcv for GAM analysis and pROC for receiver operating characteristic (ROC) analysis.

RESULTS

Characteristics of the study population

The median (5th–95th percentiles) age of the study population was 62.2 y (45.3–77.5 y), and 79.2% of subjects were men. The median (5th–95th percentiles) BMI (in kg/m²) was 26.5 (21.5–33.5), and 12.5% of subjects used vitamin B-containing supplements. Furthermore, 24.3% of subjects were current smokers, 11.9% of subjects had diabetes, and 6.3% of subjects had CRP concentrations >10 mg/dL. Additional characteristics are shown in **Table 1**.

Predictors of kynurenines

Associations of kynurenines with PLP, tryptophan, riboflavin, smoking eGFR, BMI, CRP, KTR, and neopterin are shown in **Figure 2**. Association strengths were assessed by using a Spearman's correlation adjusted for age, sex, and center (simple model 1) and with additional adjustment for all variables shown (multiple adjustment; model 2). PLP was positively associated with KA, AA, XA (both models), and HAA (only model 1) and negatively associated with HK (both models) and kynurenine (only model 2). The strongest associations with PLP (according to model 1) were found for HK ($\rho = -0.18$) and XA

TABLE 1

Baseline characteristics of the study population ($n = 2628$)¹

Characteristics	Values
Sex (M) [n (%)]	2082 (79.2)
Age (y)	62.2 (45.3–77.5) ²
BMI (kg/m ²)	26.5 (21.5–33.5)
Diabetes mellitus [n (%)]	314 (11.9)
Current smoker [n (%)]	638 (24.3)
Vitamin supplement user [n (%)]	329 (12.5)
Creatinine (μ mol/L)	73.4 (53.0–103)
CRP (mg/L)	1.7 (0.3–12.0)
KTR (nmol/ μ mol)	23.8 (15.8–39.4)
Neopterin (nmol/L)	7.8 (5.2–14.5)
PLP (nmol/L)	39.9 (18.6–101)
Riboflavin (nmol/L)	11.1 (4.5–44.3)
Tryptophan (μ mol/L)	68.0 (47.3–92.5)
Kynurenine (μ mol/L)	1.7 (1.1–2.6)
KA (nmol/L)	48.5 (26.0–92.6)
AA (nmol/L)	13.8 (7.7–26.8)
HK (nmol/L)	29.1 (15.3–58.8)
XA (nmol/L)	14.2 (6.1–30.5)
HAA (nmol/L)	34.6 (16.0–67.1)

¹AA, anthranilic acid; CRP, C-reactive protein; HAA, 3-hydroxyanthranilic acid; HK, 3-hydroxykynurenine; KA, kynurenic acid; KTR, kynurenine:tryptophan ratio; PLP, pyridoxal 5'-phosphate; XA, xanthurenic acid.

²Median; 5th–95th percentiles in parentheses (all such values).

($\rho = 0.18$). The strongest predictors for most kynurenines were tryptophan, eGFR, KTR, and neopterin.

Predictors of HK:KA, HK:AA, HK:XA, and HK:HAA

HK:KA, HK:AA, HK:XA, and HK:HAA ratios were modeled similarly to individual kynurenines (Figure 2, bottom). PLP was the strongest predictor of HK:KA, HK:AA, and HK:XA with

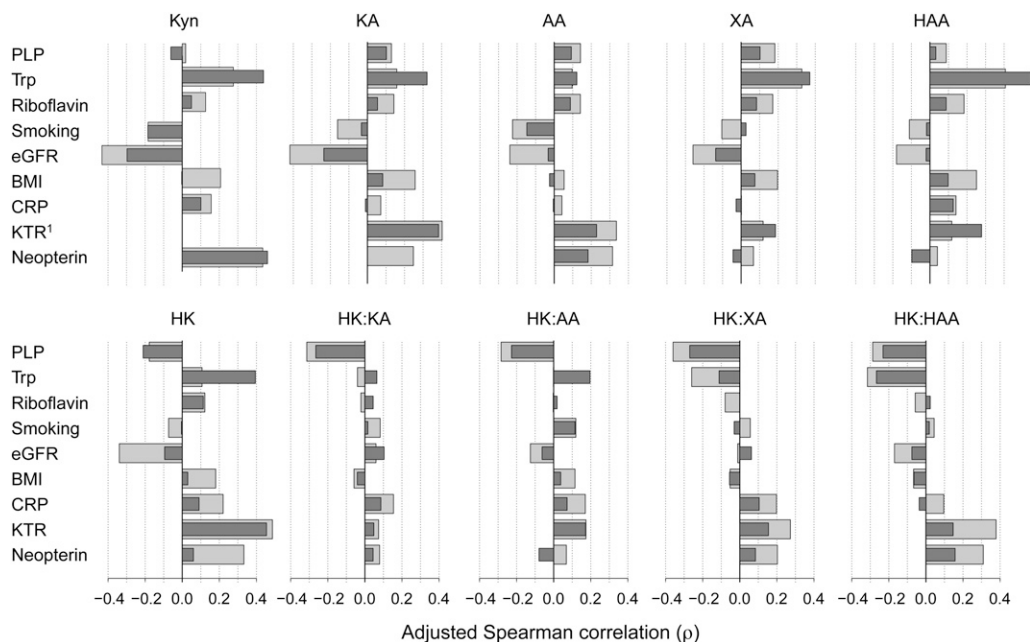


FIGURE 2. Modeling of Kyns and ratios HK:KA, HK:AA, HK:XA, and HK:HAA by Spearman's correlation. Correlations were adjusted for age, sex, and center (light-gray bars) and, in addition, for all variables shown (dark-gray bars). Coefficients >0.06 were significant at $P < 0.01$. ¹KTR was not included when modeling Kyn. AA, anthranilic acid; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; HAA, 3-hydroxyanthranilic acid; HK, 3-hydroxykynurenine; KA, kynurenic acid; KTR, kynurenine:tryptophan ratio; Kyn, kynurenine; PLP, pyridoxal 5'-phosphate; Trp, tryptophan; XA, xanthurenic acid.

$\rho = -0.31, -0.28, \text{ and } -0.36$, respectively, and remained the strongest predictor of these ratios after multiple adjustment. PLP was also a relatively strong predictor of HK:HAA ($r = -0.29$). Compared with HK, the ratios showed a stronger inverse association with PLP but no or weaker associations with tryptophan, eGFR, and inflammatory markers. In particular, HK:KA showed only weak associations with variables other than PLP.

Dose-response relations

The dose-response relation of kynurenines and HK:KA, HK:AA, HK:XA, and HK:HAA ratios with PLP were analyzed by using GAM regression. Nonlinear associations were shown for KA, XA, HK, and all 4 ratios (Figure 3). Stronger associations toward the lower end of the PLP-distribution were a common feature. We also analyzed associations of HK:KA, HK:AA, HK:XA, and HK:HAA with PLP in strata on the basis of tertiles of CRP, KTR, and neopterin. Relations were similar, although slightly stronger, in the higher tertiles of inflammatory markers (results not shown). However, differences between strata were not significant after multiple adjustments (all P -interaction > 0.01).

ROC analysis

We used ROC analysis to assess the sensitivity compared with specificity for correctly classifying low plasma PLP (defined as less than the fifth percentile or 18.6 nmol/L) for all kynurenines and ratios. AUCs (95% CIs) were 0.78 (0.74, 0.82) for HK:HAA, 0.78 (0.73, 0.82), for HK:XA, 0.74 (0.69, 0.79) for HK:KA, 0.67 (0.61, 0.72) for HK:AA, 0.65 (0.59, 0.70) for HK, and 0.65 (0.61, 0.70) for XA. The other kynurenines had AUCs < 0.65 . Ratios HK:HAA, HK:XA, and HK:KA all had a better AUC than did HK ($P < 0.001$ for all comparisons)

Effects of treatment with pyridoxine for 28 d

The change in kynurenines and ratios after oral administration of 40 mg pyridoxine hydrochloride for 28 d as a function of baseline plasma PLP was evaluated by GAM. HK and all ratios decreased, whereas all other kynurenines increased after pyridoxine treatment. For HK:HAA, the decrease ranged from approximately -60% at low to -15% at high baseline PLP. The corresponding numbers were -50% to 5% for HK:XA, and -45% to 0% for HK (Figure 4). See supplemental Figure 1 under "Supplemental data" in the online issue for changes for all kynurenines and ratios.

Associations of PLP, kynurenines, and ratios with age

Average plasma PLP concentrations decreased after ~ 55 – 60 y of age. However, in supplement users ($n = 329$; 12.5%), plasma PLP increased (P -interaction < 0.001). When we restricted the analysis to nonsupplement users, we observed increases in HK:XA and HK:HAA that mirrored the decrease in PLP (Figure 5). The analysis of individual kynurenines showed that kynurenine, KA, AA, and HK increased, whereas XA and HAA decreased slightly with age (see supplemental Figure 2 under "Supplemental data" in the online issue). All analyses were adjusted for sex, center, eGFR, CRP, and neopterin.

DISCUSSION

Principal findings

Kynurenines KA, AA, XA, and HK were significantly, but mostly weakly, associated with plasma PLP in simple and multiple-adjusted models. By comparison, HK:KA, HK:XA, HK:AA, and HK:HAA ratios showed considerably stronger associations with PLP, with correlation coefficients that ranged from -0.28 to

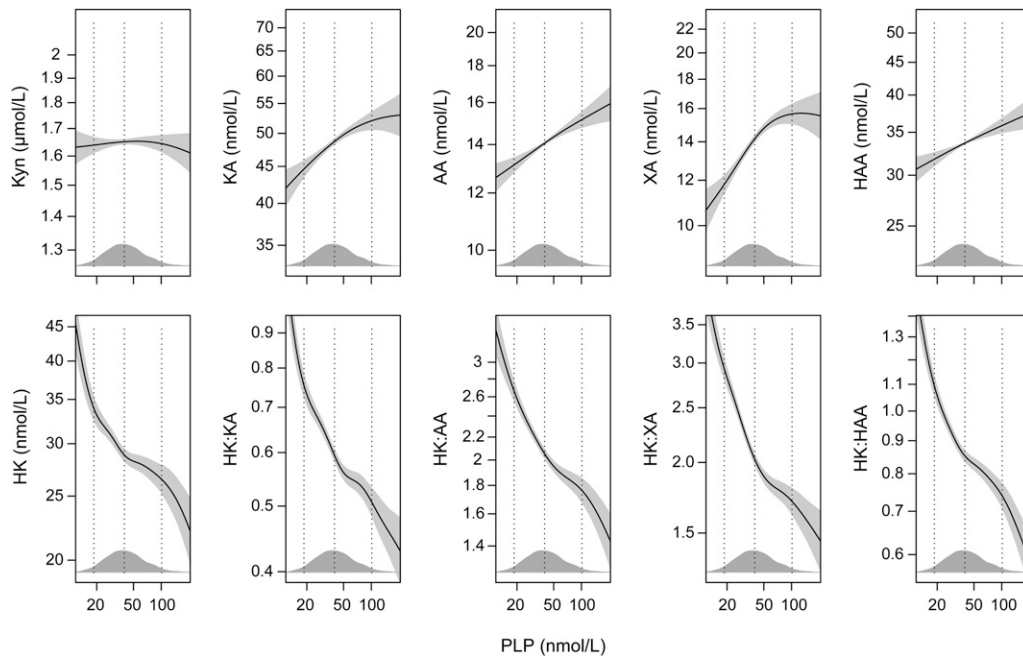


FIGURE 3. Associations (95% CIs) of Kyns and ratios HK:KA, HK:AA, HK:XA, and HK:HAA with PLP. Associations were modeled by GAM adjusted for age, sex, and center. Shaded areas indicate 95% CIs. y axes span 2 SDs of each outcome. A density plot for the distribution of PLP is included in each diagram with 5th, 50th, and 95th percentiles marked by dotted lines. AA, anthranilic acid; GAM, generalized additive models; HAA, 3-hydroxyanthranilic acid; HK, 3-hydroxykynurenine; KA, kynurenic acid; Kyn, kynurenine; PLP, pyridoxal 5'-phosphate; XA, xanthurenic acid.

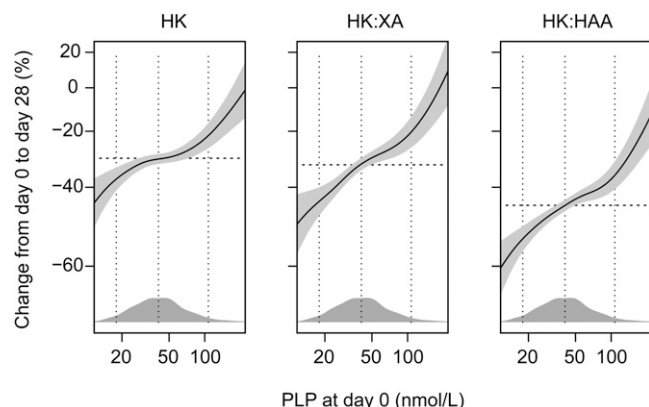


FIGURE 4. Changes (95% CIs) in HK, HK:XA, and HK:HAA after oral administration of 40 mg pyridoxine hydrochloride for 28 d compared with baseline PLP. The change (%) from days 0 to 28 was modeled by GAM adjusted for intervention group. Shaded areas indicate 95% CIs. The distribution curve for PLP at baseline is included in each diagram with 5th, 50th, and 95th percentiles denoted by dotted lines. The average response is shown by the horizontal dotted line. GAM, generalized additive model; HAA, 3-hydroxyanthranilic acid; HK, 3-hydroxykynurenine; PLP, pyridoxal 5'-phosphate; XA, xanthurenic acid.

-0.36. For all ratios, associations were nonlinear with the strongest association at the lower end of the PLP distribution. The 3 best indexes for the discrimination of low plasma PLP (less than the fifth percentile) according to ROC analysis were HK:HAA, HK:XA, and HK:KA with AUCs of 0.78, 0.78, and 0.74, respectively. The oral administration of 40 mg pyridoxine for 1 mo reduced the ratios by up to 60% depending on baseline PLP status. Finally, a decline in plasma PLP after age 60 y was paralleled by proportional increases in HK:XA and HK:HAA.

HK compared with HK-based kynurenine ratios as markers of vitamin B-6 status

Previously, we reported a sharp increase in HK at low PLP concentrations. Because the further metabolism of HK is mediated by 2 PLP-dependent enzymes, we argued that elevated HK could reflect reduced enzyme activities because of impaired

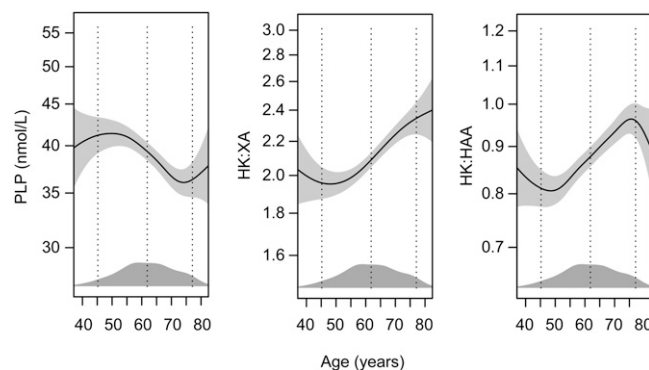


FIGURE 5. Associations (95% CIs) of PLP, HK:XA, and HK:HAA with age. Associations were modeled by GAM adjusted for sex, center, eGFR, CRP, and neopterin. Shaded areas indicate 95% CIs. y axes span 1.5 SDs of each outcome. The distribution curve for age is included in each diagram with 5th, 50th, and 95th percentiles denoted by dotted lines. CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; GAM, generalized additive model; HAA, 3-hydroxyanthranilic acid; HK, 3-hydroxykynurenine; PLP, pyridoxal 5'-phosphate; XA, xanthurenic acid.

intracellular vitamin B-6 status (17). However, we also noted that HK increased at low PLP mainly in subgroups with elevated concentrations of inflammatory markers. This association seemed to indicate that HK was a marker of vitamin B-6 status mainly during conditions of activated inflammation. In the current study, we confirmed that HK was more strongly associated with several inflammatory markers than with PLP. In contrast, PLP was the strongest predictor for HK:XA, HK:KA, and HK:AA ratios. In addition, similar inverse relations between PLP and all ratios were shown across tertiles of inflammation marker concentrations. Finally, considerable improvements in the AUC for the correct classification of plasma PLP less than the fifth percentile were shown for HK:XA, HK:HAA, and HK:KA compared with HK in ROC analyses.

Comparison of ratios

The ratio HK:HAA in urine was previously proposed as the best (most sensitive) marker of vitamin B-6 status (6, 8). However, in this study of kynurenines in plasma, HK:XA was as sensitive as HK:HAA for discriminating low plasma PLP in the ROC analysis. In addition, the overall correlation of HK:XA with PLP was stronger, and correlations with other variables mostly weaker. Motivated by the positive correlation of PLP with KA and AA, we also included HK:KA and HK:AA in the analyses. Of these ratios, HK:KA showed some interesting properties including a comparatively high AUC in the ROC analysis and only weak associations with other variables. HK:AA was the overall poorest performing marker and, therefore, is not discussed further.

Mechanisms

Studies have shown that rat liver contains considerable amounts of apokynureninase, with an enzyme activity that increases 4–5 times on addition of pyridoxine in vitro (22). Another study provided indirect evidence for a similar, normally inactive, pool of apokynureninase in humans (9). We previously reported that several days of oral supplementation with 40 mg pyridoxine hydrochloride resulted in decreased HK and increased KA, AA, and HAA (17). In the current study, we, in addition, showed that the effect of vitamin B-6 treatment was dependent on baseline plasma PLP. Together, these results show that activities of KAT as well as kynureninase are sensitive to changes in vitamin B-6 status. Decreases in XA and HAA indicate that the balance between the production and removal is altered when vitamin B-6 status is low. None of the reactions immediately downstream of HAA are PLP dependent; therefore the rate of removal of HAA should essentially be unaffected by vitamin B-6 status. XA is largely removed through the excretion in urine as reflected by the negative association between XA and eGFR. The ratio HK:KA had properties that resembled those of HK:XA. This result can be explained by KA and XA sharing the same enzyme, KAT, for production and their similar dependence on eGFR. The finding also implied that the flux through the (FAD-dependent) kynurenine monooxygenase, which converts kynurenine to HK, was unaffected by vitamin B-6 status, as confirmed by the lack of (or weak) association between kynurenine and PLP.

Substrate product ratios in plasma as indexes of enzyme activity

Among the kynurenines, HK had the most interesting properties in terms of being a candidate marker of vitamin B-6 status. However, according to metabolic control analysis, a change in the activity of a single enzyme would lead to changes in both substrate and product (23). Thus, by combining information about the product as well as the substrate, a more complete description of the state of the enzyme is obtained. Ratios have the additional advantage of eliminating influences shared by metabolites. In the present case, associations with several variables, including inflammatory markers and kidney function, were attenuated.

PLP and kynurenines versus age

Plasma PLP concentrations were previously shown to be stable in adults up to ~60 y and then declined (24). The reason for this age relation is unclear but could be related to inadequate vitamin B-6 intake or increased inflammation (24). A similar PLP-age relation was shown in this study when the analysis was confined to nonsupplement users. Notably, in adjusted analyses, the association of HK:XA and HK:HAA with age was similar and opposite to that of PLP. This result may suggest that HK:XA and HK:HAA ratios are sufficiently sensitive to monitor nutritional or physiologic effects on plasma PLP that occurs in older age.

Strength and limitations

The main limitation of the study was the cross-sectional design for most of the main results. Another limitation was the reliance on a single parameter, plasma PLP, as the reference indicator for vitamin B-6 status, as this was the only option available in stored plasma samples. Most blood samples were taken nonfasting, and we had too limited data to accurately assess the influence of prandial status. Finally, kynurenines are also determined by tryptophan dioxygenase, which is activated by tryptophan and glucocorticoids. Although this could conceivably influence the kynurenine–vitamin B-6 relation, we did not have specific data to assess this possibility.

Strengths of the study include a large and homogenous study population and measurements of most biomarkers in a single laboratory by a multiplexing method including B vitamins and all kynurenines (16). The stability of plasma metabolites according to sample handling and storage conditions has been validated (20). We also had access to longitudinal data that allowed for the assessment of the effect of pyridoxine intervention on kynurenines and ratios. Finally, we excluded WENBIT participants diagnosed with acute coronary syndrome, and the resulting study cohort had close to normal concentrations of inflammatory biomarkers. Therefore, the results may be applicable to healthy populations of a similar age.

In conclusion, we have shown that low plasma PLP concentrations are associated with high values of HK:XA, HK:HAA, HK:KA, and HK:AA ratios in plasma. HK:XA and HK:HAA are substrate-product pairs of the PLP-dependent enzymes KAT and kynureninase, respectively. The ratios may reflect the respective amount of PLP-bound enzyme and, therefore, intracellular PLP availability. HK:XA appeared the best candidate to be used as a marker of vitamin B-6 status on the basis of its correlation with

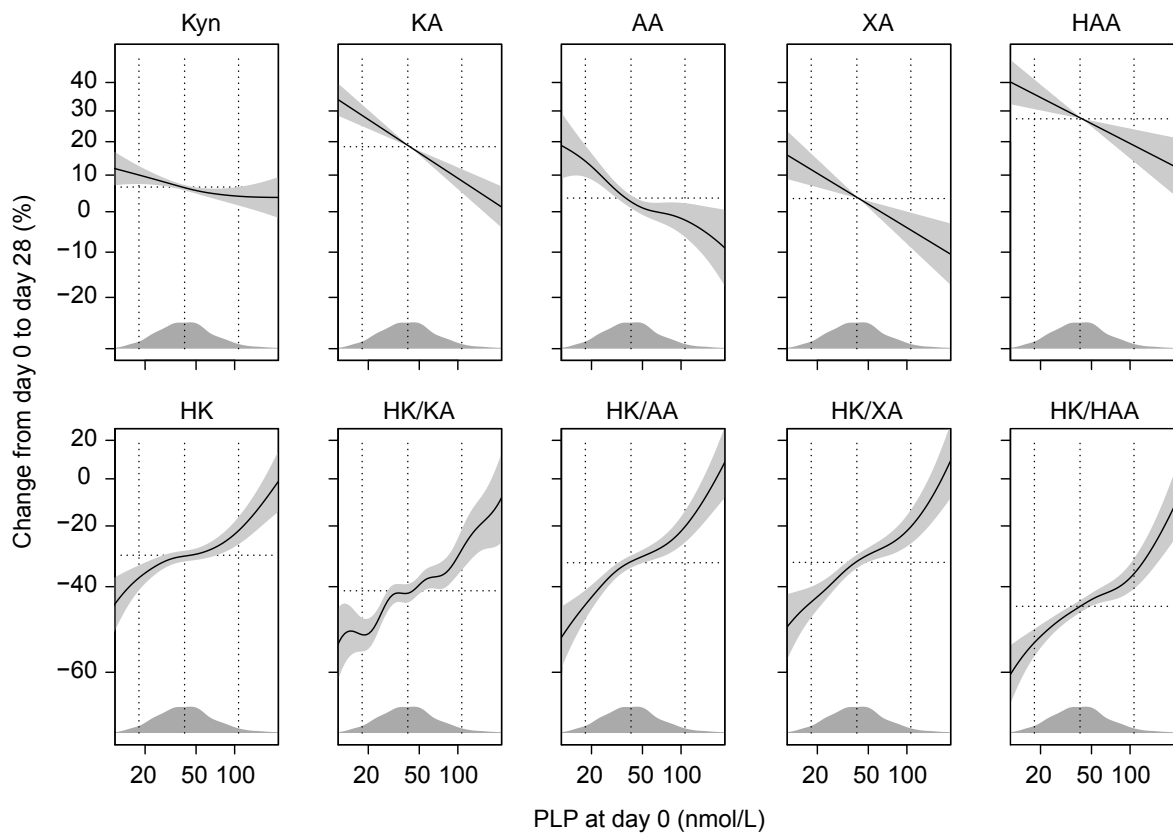
PLP, its performance for correctly classifying low plasma PLP in the ROC analysis, and relatively modest association with other variables that may represent potential confounders in clinical studies. In addition, the ratios HK:KA and HK:HAA also showed properties that warrant further validation. Applications of these indexes as markers of functional vitamin B-6 status could include the clinical setting as well as research purposes on the basis of established biorepositories.

The authors' responsibilities were as follows—AU, ON, and PMU: study concept and design; ØM: acquisition of data; AU: analysis of data; AU and DT: drafting of the manuscript; AU, DT, PMU, ØM, SJPM, and ON: critical revision of the manuscript for important intellectual content; AU: primary responsibility for the final content of the manuscript; and all authors: reading and approval of the final manuscript. AlphaPharma Inc played no role in the design, implementation, analysis, and interpretation of the study. None of the authors had a conflict of interest.

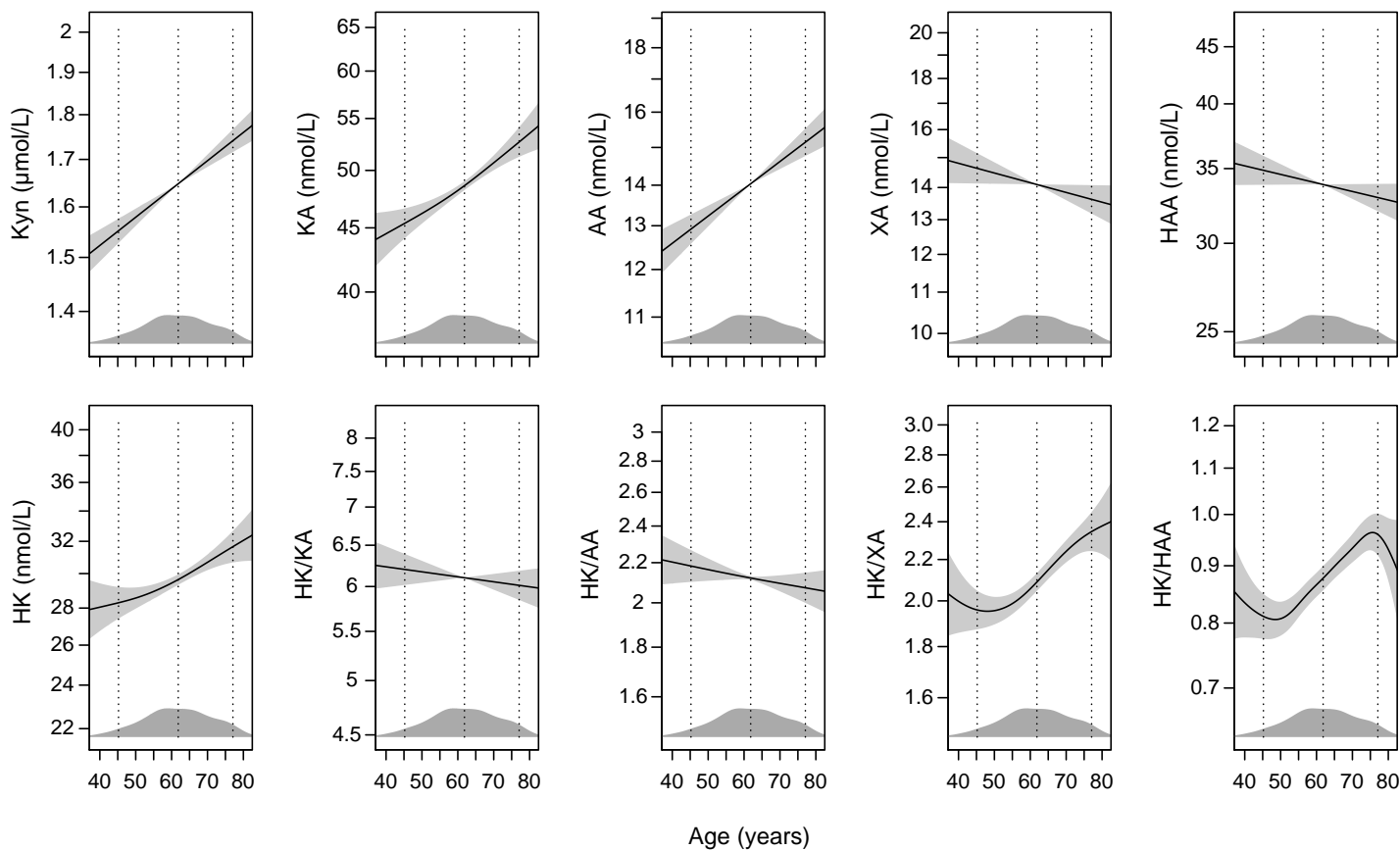
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Supplemental figure 1 Change in kynurenines, HK/KA, HK/AA, HK/XA and HK/HAA after oral administration of 40 mg pyridoxine hydrochloride for 28 days vs. baseline PLP. Change (%) from day 0 to day 28 was modeled by GAM adjusted for intervention group. Shaded areas indicate 95% confidence intervals. The distribution curve for PLP at baseline is included in each diagram with 5th, 50th, and 95th percentiles denoted by dotted lines. The average response is shown by the horizontal dotted line. Abbreviations: GAM, generalized additive models; HAA, 3-OH anthranilic acid; HK, 3-OH kynurenine; XA, xanthurenic acid.



Supplemental figure 2 Association of kynurenines, HK/KA, HK/AA, HK/XA, and HK/HAA with age. Associations were modeled by GAM adjusted for sex, centre, eGFR, CRP and neopterin. Shaded areas indicate 95% confidence intervals. The y-axes span 1.5 standard deviations of each outcome. The distribution curve for age is included in each diagram with 5th, 50th and 95th percentiles denoted by dotted lines. Abbreviations: CRP, C-reactive protein; eGFR, estimated glomerular filtration; GAM, generalized additive models; HAA, 3-OH anthranilic acid; HK, 3-OH kynurenine; XA, xanthurenic acid.