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Tryptophan catabolites as metabolic markers of vitamin B-6 status evaluated in cohorts of healthy adults and cardiovascular patients

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ABSTRACT

Background: Vitamin B-6 status is routinely measured as pyridoxal 5′-phosphate (PLP) in plasma. Low concentrations of PLP are associated with rheumatic, cardiovascular, and neoplastic diseases. We have previously shown that vitamin B-6 status affects the kynurenine (Kyn) pathway of tryptophan (Trp) catabolism.

Objective: This study aimed to comprehensively evaluate the use of Kyns as potential markers of functional vitamin B-6 status across 2 large cohorts.

Methods: We measured circulating concentrations of the first 6 metabolites in the Trp catabolic pathway by LC–MS-MS in the community-based Hordaland Health Study (HUSK; $n = 7017$) and cardiovascular patient–based Western Norway Coronary Angiography Cohort (WECAC; $n = 4161$). Cross-sectional and longitudinal associations of plasma PLP with Kyns were estimated using linear and nonlinear regression–based methods.

Results: 3′-Hydroxykynurenine (HK), a substrate, and all 4 products formed directly by the PLP-dependent enzymes kynurenine transaminase and kynureninase contributed to the explanation of circulating PLP in multivariable-adjusted regression models. The construct HK:(kynurenic acid + xanthurenic acid + 3′-hydroxyanthranilic acid + anthranilic acid), termed HK ratio (HKr), was related to plasma PLP with standardized regression coefficients (95% CIs) of −0.47 (−0.49, −0.45) and −0.46 (−0.49, −0.43) in HUSK and WECAC, respectively. Across strata of cohort and sex, HKr was 1.3- to 2.7-fold more sensitive, but also 1.7- to 2.9-fold more specific to changes in PLP than a previously proposed marker, HK:xanthurenic acid. Notably, the association was strongest at PLP concentrations < ∼20 nmol/L, a recognized threshold for vitamin B-6 deficiency. Finally, PLP and HKr demonstrated highly sex-specific and corroborating associations with age.

Conclusions: The results demonstrate that by combining 5 metabolites in the Kyn pathway into a simple index, HKr, a sensitive and specific indicator of intracellular vitamin B-6 status is obtained. The data also underscore the merit of evaluating alterations in Kyn metabolism when investigating vitamin B-6 and health. *Am J Clin Nutr* 2020;111:178–186.

Keywords: vitamin B-6, nutritional status, biomarker, inflammation, metabolites, kynurenines, tryptophan

Introduction

The involvement of vitamin B-6 in human metabolism includes the synthesis and interconversion of amino acids, neurotransmitters, nucleic acids, heme, and lipids. Vitamin B-6 also plays an important role in energy homeostasis through glycogen degradation and gluconeogenesis. The versatility of pyridoxal 5′-phosphate (PLP), the active form of vitamin B-6, is underscored by its use as a coenzyme in all the major enzyme classes except for ligases (1). Both vitamin B-6 intake and plasma indicators of vitamin B-6 status have been associated with clinical conditions including, but not limited to, rheumatoid, cardiovascular, and neoplastic diseases as well as mortality in cross-sectional and prospective studies (2–5).

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Supplemental Figures 1–3 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: AA, anthranilic acid; CAD, coronary artery disease; CRP, C-reactive protein; GAM, generalized additive model; HAA, 3′hydroxyanthranilic acid; HK, 3′hydroxykynurenine; HKr, HK ratio; HUSK, Hordaland Health Study; KA, kynurenic acid; KAT, kynurenine transaminase; KMO, kynurenine monooxygenase; KTR, kynurenine:tryptophan ratio; kyn, kynurenine; KYNU, kynureninase; PLP, pyridoxal 5′-phosphate; Trp, tryptophan; WECAC, Western Norway Coronary Angiography Cohort; WENBIT, Western Norway B-Vitamin Intervention Trial; XA, xanthurenic acid.

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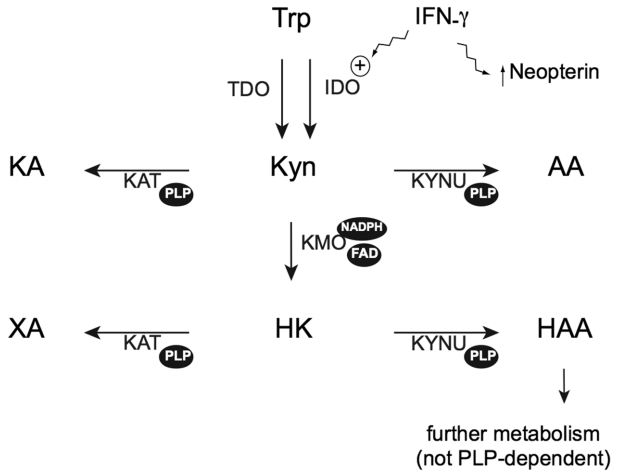

FIGURE 1 Trp metabolism through the Kyn pathway. Enzymes and cofactors are shown. IDO is activated by inflammatory stimuli including IFN-γ, which also stimulates macrophages to produce neopterin. The immediate product of TDO and IDO, formylkynurenine, is not shown. AA, anthranilic acid; HAA, 3′hydroxyanthranilic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

One of the earliest described indicators of low vitamin B-6 status was the increased excretion of the tryptophan (Trp) catabolite xanthurenic acid (XA) in urine after a Trp load (6). Subsequently, a number of metabolites along the kynurenine (Kyn) pathway of Trp catabolism were found to be increased in the urine of vitamin B-6–deficient humans, including the ratio of 3′hydroxykynurenine to 3′hydroxyanthranilic acid (HK:HAA) (7, 8). An overview of Trp metabolism and its 2 PLP-dependent steps is shown in **Figure 1**. Methods and protocols for quantification of these and other functional markers of vitamin B-6 status are often cumbersome, however, and have largely been abandoned since sensitive and precise measurements of plasma PLP became available (9, 10). Although plasma PLP is accepted as an indicator of nutritional vitamin B-6 status, PLP has been found to be redistributed from plasma to tissues, e.g., erythrocytes and liver, during inflammation, which may complicate the interpretation of plasma PLP in observational studies (3, 11).

A decade ago we expanded an assay for the quantification of the B-6 vitamers (PLP, pyridoxal, and 4′-pyridoxic acid) in serum or plasma to also include Trp and the first 6 metabolites of the Trp degradation pathway. In a cohort of suspected coronary artery disease (CAD) patients we noted that HK was markedly increased at plasma PLP concentrations <20 nmol/L, a cutoff suggested to indicate vitamin B-6 deficiency (12). In a follow-up study we evaluated substrate:product ratios of the 2 PLP-dependent enzymes kynurenine aminotransferase (KAT) and kynureninase (KYNU) and found that HK:xanthurenic acid (HK:XA) exhibited both increased sensitivity and specificity for PLP compared with HK alone (13). Subsequently, this and other Kyn ratios have been associated with increased risk of cancers of the lung (14) and colon (15), mortality in renal transplant recipients (16), and with treatment efficacy in rheumatoid patients (in a forthcoming article (17)).

Previously, we evaluated Kyns in a cohort of confirmed and suspected CAD patients (13). The objective of the present study was to perform an in-depth exploration of the concept of Kyns as metabolic markers of vitamin B-6 status and to extend and diversify the population base to include more CAD patients as well as participants from a large community-based cohort, the Hordaland Health Study (HUSK).

Methods

Study populations

HUSK is a community-based longitudinal observational study whose baseline measurements were conducted during 1997–1999 (http://husk-en.w.uib.no). Details of the study design and methodology have been described elsewhere (18, 19). The HUSK cohort, as used here, encompasses 7050 men and women who were born during 1925–1927 or 1950–1951 and living in or adjacent to the city of Bergen, Norway. After exclusion of 126 participants with missing data on PLP and Kyns, cross-sectional data for 6924 participants (3062 men and 3862 women) were included in the present analyses. The Western Norway Coronary Angiography Cohort (WECAC) consists of 4164 patients that underwent elective coronary angiography due to suspected stable angina pectoris between 2000 and 2004 (20). About two-thirds of these patients participated in the Western Norway B-Vitamin Intervention Trial (WENBIT), which evaluated the lowering of plasma homocysteine by oral B-vitamin treatment to prevent future cardiovascular events. The 4 treatment groups consisted of *1*) 0.8 mg folic acid, 0.4 mg cyanocobalamin, and 40 mg pyridoxine; *2*) 0.8 mg folic acid and 0.4 mg cyanocobalamin; *3*) 40 mg pyridoxine; and *4*) placebo in a 2 × 2 factorial design. WENBIT is described in detail elsewhere (21). After exclusion of 45 participants with missing data on PLP and Kyns, cross-sectional data for 4119 participants (2960 men and 1159 women) were included in the present analyses. In addition, for the WENBIT study participants, we used data also from the first study visit to evaluate the association of changes in PLP with changes in Kyns across 28 d. Complete data for 2508 participants were available for this analysis. Participant flowcharts for HUSK and WECAC are available as **Supplemental Figures 1** and **2**, respectively.

Sociodemographic and anthropometric variables

Sociodemographic and anthropometric data were obtained by self-administered questionnaires (HUSK) or interview (WECAC). Smoking status was based on self-reported smoking habits corrected by plasma cotinine, i.e., patients initially classified as nonsmokers but with plasma cotinine ≥85 nmol/L (22) were reclassified as smokers. Height and weight were measured using standardized protocols, and BMI was calculated as kg/m^2.

Laboratory analyses

Nonfasting blood samples were collected into tubes containing EDTA, kept on ice before centrifugation (within 3 h), and stored at –80°C before analysis. Plasma concentrations of PLP, Trp, Kyns, neopterin, cotinine, and creatinine were quantified by LC/tandem MS at Bevital, Bergen, Norway (www.bevital.no) (23, 24). C-reactive protein (CRP) was measured in serum using an ultrasensitive immunoassay, Behring nephelometer II system N Latex CRP mono (Behring Diagnostics) (WECAC), or in

TABLE 1 Characteristics of the study population[1]

	HUSK			WECAC		
	Men	Women	P^2	Men	Women	P^2
n (%)	3062 (44.2)	3862 (55.8)		2960 (72.0)	1159 (28.1)	
Age 46–47 y, *n* (%)	1623 (44.4)	2033 (55.6)		—	—	
Age 70–72 y, *n* (%)	1439 (44.0)	1829 (56.0)		—	—	
Age, y	—	—		61 [54–69]	64 [56–71]	<0.001
Current smoker, *n* (%)	906 (29.6)	1044 (27.0)	0.02	1003 (33.9)	305 (26.3)	<0.001
BMI, kg/m^2	25.8 [23.9–27.9]	24.9 [22.5–28.0]	<0.001	26.4 [24.4–28.7]	26.1 [23.3–29.4]	0.009
Creatinine, mmol/L	88.5 [81.3–96.8]	73.7 [67.7–80.4]	<0.001	77.4 [68.7−86.8]	64.8 [57.4–73.7]	<0.001
PLP, nmol/L	50.5 [36.6–72.4]	49.8 [34.6–78.9]	0.80	42.0 [29.9−59,6]	39.7 [28.6–60.8]	0.10
Trp, mmol/L	70.5 [62.0–79.9]	64.3 [56.0–73.5]	<0.001	71.4 [62.2−80.9]	66.5 [57.0–76.1]	<0.001
Kyn, mmol/L	1.59 [1.35–1.91]	1.45 [1.21–1.76]	<0.001	1.70 [1.41−2.01]	1.63 [1.35−2.02]	0.005
HK, nmol/L	31.9 [25.5–39.9]	32.5 [25.8–40.6]	0.05	30.1 [23.7−38.8]	32.8 [25.2−42.8]	<0.001
KA, nmol/L	48.9 [39.1–62.3]	42.5 [33.8–55.1]	<0.001	50.1 [38.8−64.9]	43.0 [33.1−54.8]	<0.001
XA, nmol/L	16.9 [12.2–22.8]	14.7 [10.6–20.0]	<0.001	15.1 [10.8−20.7]	12.7 [8.8−18,2]	<0.001
AA, nmol/L	14.4 [11.7–18.3]	14.0 [11.3–17.6]	0.001	14.3 [11.4−18.3]	14.5 [11.3−18.3]	0.64
HAA, nmol/L	35.2 [27.7–44.9]	31.9 [25.1–40.8]	<0.001	35.9 [27.0−46.9]	30.7 [23.8−39.8]	<0.001
HK:XA (no units)	1.88 [1.45–2.49]	2.19 [1.62–3.07]	<0.001	1.99 [1.54−2.62]	2.48 [1.84−3.62]	<0.001
HKr × 100 (no units)	26.9 [22.5–32.1]	30.1 [24.7–36.9]	<0.001	25.5 [21.1−31.2]	31.0 [24.9−39.3]	<0.001
CRP, mg/L	1.62 [0.75−3.58]	1.54 [0.64–3.65]	0.03	1.77 [0.86−3.54]	1.80 [0.91−3.98]	0.07
KTR, nmol/μmol	22.6 [18.7–27.9]	22.5 [18.3−28.0]	0.52	23.5 [19.7−28.5]	24.8 [20.4−30.6]	<0.001
Neopterin, nmol/L	7.5 [6.3–9.2]	7.8 [6.5−9.4]	<0.001	7.9 [6.5−9.9]	8.9 [7.1−11.4]	<0.001

[1] Values are medians [25th–75th percentile] unless otherwise indicated. AA, anthranilic acid; CRP, C-reactive protein; HAA, 3′hydroxyanthranilic acid; HK, 3′hydroxykynurenine; HKr, ratio of HK to (KA + XA + AA + HAA); HUSK, Hordaland Health Study; KA, kynurenic acid; KTR, kynurenine:Trp ratio; Kyn, kynurenine; PLP, pyridoxal 5′-phosphate; Trp, tryptophan; WECAC, Western Norway Coronary Angiography Cohort; XA, xanthurenic acid.

[2] Mann–Whitney *U* test or Fisher's exact test for the difference between men and women.

plasma with an immuno-MALDI–based assay (HUSK) (25). Further details concerning handling and storage of blood samples before analysis (WECAC) have been described previously (20, 26, 27).

Statistical methods

All continuous variables were log-transformed before inclusion in parametric regression models to satisfy the criterion of normality of residuals in linear regression analysis. Differences by gender were evaluated by the Mann–Whitney *U* test for continuous data and by Fisher's exact test for categorical data. The correlations between Kyns were estimated by Pearson's *r* adjusted for age and sex. Linear and nonlinear associations between vitamin B-6 markers and PLP were evaluated by multivariable linear regression, generalized additive models (GAMs), and segmented regression. Subcohorts were based on dichotomous variables, or, if continuous, above compared with below the median. Data were divided into low and high inflammation according to the median of the product of CRP, neopterin, and the Kyn:Trp ratio (KTR). Predictors of vitamin B-6 markers were evaluated using "relative importance regression." This method combines multiple linear regression with the algorithm "lmg" as described elsewhere (28). Briefly, the algorithm evaluates all possible models and all sequences for addition to a regression model that can be applied to a given set of predictors (regressors). The impact of each regressor is then averaged over these models using the percentage explained variance as a metric. The predictors included age, smoking (current/no current), BMI, creatinine, CRP, neopterin, KTR, and PLP, and analyses were performed separately for men and women. From the output of these analyses we calculated performance indexes using the following definitions: sensitivity, the amount of variation in the outcome explained by PLP; specificity, the ratio of this number to the total explained variation; and performance as sensitivity ∗ specificity. Notably, these terms should not be confused with similar terms used in receiver operating curve analysis. We used R for Macintosh version 3.5.2 (The R Foundation for Statistical Computing, www.r-project.org) for all statistical calculations, with the R-packages "mgcv" for GAMs, "segmented" for segmented regression, and "relaimpo" for the multiple linear regression–based assessment of relative importance of predictors.

Results

Characteristics of the study populations

The HUSK cohort consisted of 2 distinct age groups: 46–47 y (52.8%) and 70–72 y, with ∼56% women in each age group. In WECAC, the median (25th–75th percentile) age was 61 (54–69) y for men, and 64 (56–71) y for women, with 28.1% women. The concentrations of Trp and the Kyns were similar in HUSK and WECAC (**Table 1**). In both cohorts, the concentrations of Trp, Kyn, kynurenic acid (KA), XA, and HAA were higher in men than in women. PLP concentrations were similar across genders, but both HK:XA and the HK ratio (HKr) [equal to HK:(KA + XA + HAA + anthranilic acid)] were higher in women (Table 1). In WECAC, 2125 patients (52%) had previously established cardiovascular disease and 3082 (75%) had ≥1 stenotic vessel based on coronary angiography.

TABLE 2 Linear associations between vitamin B-6 markers and pyridoxal 5′-phosphate[1]

	HUSK	WECAC
Single kynurenines		
HK	–0.26 (–0.29, –0.24)	–0.25 (–0.28, –0.22)
KA	0.13 (0.10, 0.15)	0.13 (0.10, 0.16)
XA	0.13 (0.10, 0.15)	0.17 (0.14, 0.20)
AA	0.07 (0.04, 0.09)	0.10 (0.07, 0.13)
HAA	0.17 (0.15, 0.20)	0.12 (0.09, 0.15)
Ratios flanking KAT		
HK:KA	–0.38 (–0.41, –0.36)	–0.37 (–0.40, –0.35)
HK:XA	–0.33 (–0.35, –0.30)	–0.41 (–0.44, –0.38)
HK_{KAT} = HK:(KA + XA)	–0.41 (–0.43, –0.39)	–0.41 (–0.44, –0.38)
Ratios flanking KYNU		
HK:AA	–0.28 (–0.30, –0.26)	–0.29 (–0.32, –0.27)
HK:HAA	–0.43 (–0.45, –0.41)	–0.38 (–0.41, –0.35)
HK_{KYNU} = HK:(AA + HAA)	–0.43 (–0.46, –0.41)	–0.40 (–0.43, –0.38)
Ratio flanking both enzymes		
HKr = HK:(KA + XA + AA + HAA)	–0.47 (–0.49, –0.45)	–0.46 (–0.49, –0.43)

[1]Numbers are standardized regression coefficients (95% CIs) adjusted for age and sex. All associations were significant at $P < 0.0001$. AA, anthranilic acid; HAA, 3′hydroxyanthranilic acid; HK, 3′hydroxykynurenine; HKr, HK ratio; HUSK, Hordaland Health Study; KA, kynurenic acid; KAT, kynurenine transaminase; KYNU, kynureninase; WECAC, Western Norway Coronary Angiography Cohort; XA, xanthurenic acid.

Initial exploration of the relation between PLP and Kyns

We modeled PLP by linear regression and stepwise selection using the Kyns downstream of Kyn as candidate predictors while keeping age and sex as fixed covariates. In both HUSK and WECAC, HK was selected as the first (and only negative) predictor of PLP followed by all 4 of XA, KA, HAA, and anthranilic acid (AA) (positive predictors). In unadjusted analyses, we confirmed that the proportions of KA, XA, HAA, and AA all increased whereas HK decreased across quartiles of PLP. The correlations (Pearson's r adjusted for age and sex) of KA, XA, HAA, and AA with their sum were 0.85, 0.76, 0.49, and 0.79, respectively in HUSK, and 0.88, 0.80, 0.52, and 0.71 in WECAC. Inspired by these results, HKr = HK:(KA + XA + AA + HAA) was constructed as a candidate marker of vitamin B-6 status. We also included ratios aimed at specifically characterizing KAT denoted HK_{KAT} = HK:(KA + XA), and KYNU: HK_{KYNU} = HK:(AA + HAA). In what follows we will refer to Kyns, either singly or in combination, as (potential) functional vitamin B-6 markers.

Linear associations between PLP and selected vitamin B-6 markers

We evaluated the linear association of PLP with vitamin B-6 markers by multiple linear regression adjusted for age and sex (**Table 2**). Notably, Kyn combinations (ratios) were more strongly associated with PLP than were individual metabolites, and HKr demonstrated the strongest association with PLP in both cohorts. The associations of PLP with Kyn and the ratios Kyn:KA and Kyn:AA were all weak (standardized βs > –0.14). When evaluated in strata based on sex, age, vitamin supplement use, and inflammation (both cohorts) and according to established CVD at baseline and ≥1 stenotic vessel (WECAC), HKr was consistently the best marker in terms of strength of association with PLP.

Determinants of vitamin B-6 markers

We evaluated the associations of selected vitamin B-6 markers with age; BMI; current smoking; kidney function (creatinine); inflammation, as represented by the 3 variables CRP, neopterin, and KTR; and PLP using relative importance regression stratified by sex. Results for the markers HK, HK:XA, and HKr in WECAC are shown in **Figure 2** and relative performances of the markers by cohort and sex are summarized in **Table 3**. As demonstrated in Figure 2 and Table 3, both the sensitivity and specificity for PLP increased in the direction of more complex ratios, and, again, except for specificity in WECAC females, HKr was the best-scoring marker in all strata (Table 3). In addition to the markers included in Table 3, we also evaluated a construct where the 4 downstream Kyns were standardized before summation, and another construct where we used the product instead of the sum of the 4 downstream Kyns. Compared with HKr, the performance of these alternative markers was considerably poorer by the criteria used in Table 3.

The association of vitamin B-6 markers with PLP by GAM regression

Figure 3 shows the association of HK, HK:XA, and HKr with PLP in HUSK by GAMs. Corresponding, and very similar, results were found in WECAC (**Supplemental Figure 3**). Common to all markers was a markedly stronger association at low than at normal and high PLP concentrations. Using segmented regression, we identified a breakpoint (95% CI) at 19.4 (18.1, 20.7) nmol/L PLP for the HKr–PLP association in HUSK and a similar breakpoint (95% CI) at 19.1 (17.4, 20.9) nmol/L in WECAC (Supplemental Figure 3). Close examination of the GAM curves suggested a transitional segment of intermediate sensitivity to PLP in the interval of ∼20–40 nmol/L in both cohorts, but we were unable to obtain reproducible breakpoints for a possible intermediate segment by segmented regression.

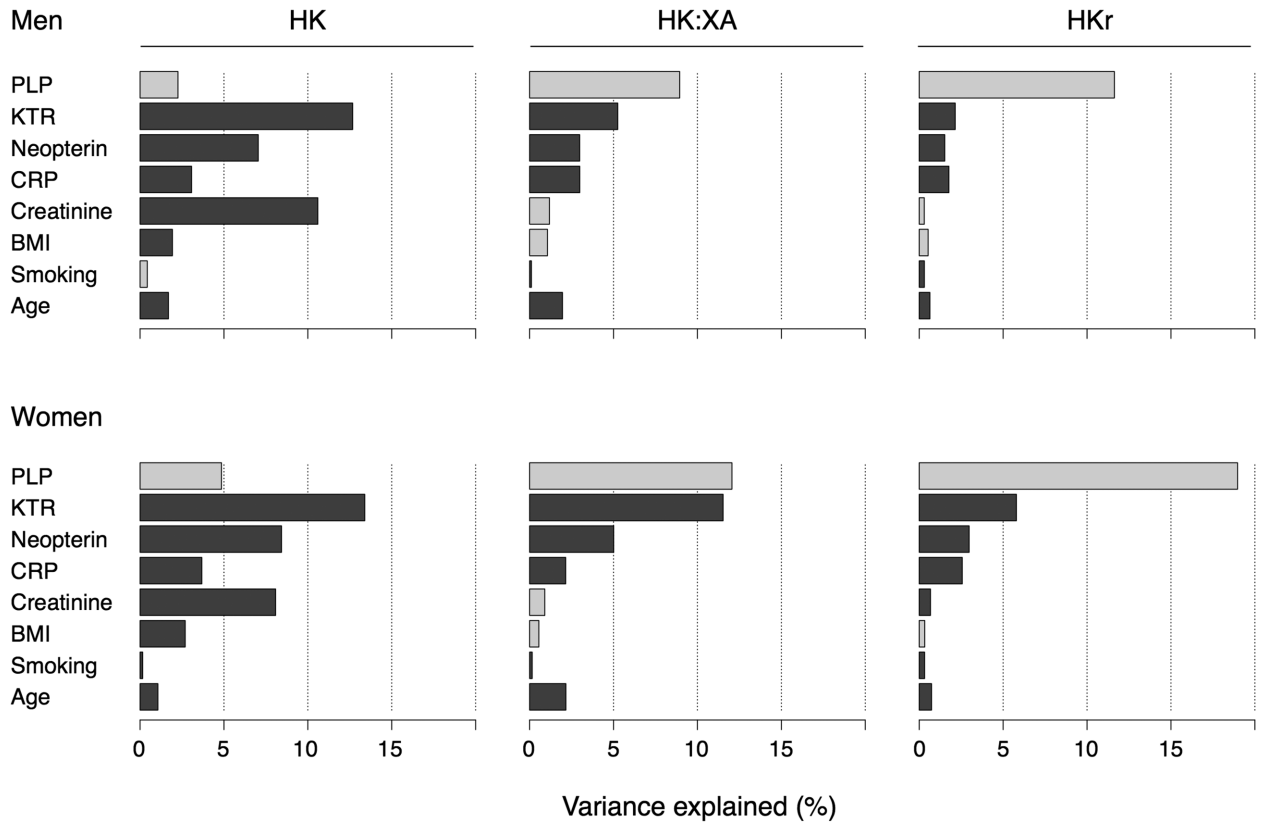

FIGURE 2 Relative importance of predictors of HK, HK:XA, and HKr in WECAC. The amount of variation in HK, HK:XA, and HKr attributable to PLP and relevant confounders is shown. Calculations were based on multiple linear regression and the "relaimpo" package in R, with R^2 as the metric for explained variation. Negative and positive associations are depicted in light gray and black, respectively. CRP, C-reactive protein; HK, 3′hydroxykynurenine; HKr, HK ratio; KTR, kynurenine:tryptophan ratio; PLP, pyridoxal 5′-phosphate; WECAC, Western Norway Coronary Angiography Center; XA, xanthurenic acid.

Longitudinal associations

For WECAC patients who participated in the WENBIT trial we had data on PLP and vitamin B-6 markers at baseline and at the day 28 study visit. Moreover, 1 arm of the 2 × 2 factorial randomized controlled trial design included a daily oral dose of 40 mg pyridoxine. Thus, we were able to assess the change in vitamin B-6 markers according to both natural variation in PLP (across 28 d) and pyridoxine treatment. **Figure 4** shows

TABLE 3 Performance characteristics of selected markers of vitamin B-6 status[1]

	HUSK			WECAC		
	Sensitivity (se)	Specificity (sp)	Performance (se ∗ sp)	Sensitivity (se)	Specificity (sp)	Performance (se ∗ sp)
Men						
HK	2.4	7.4	0.2	2.3	5.7	0.1
HK:XA	6.5	22.4	1.5	8.9	36.5	3.3
HK_{KAT} = HK:(KA + XA)	9.3	54.9	5.1	9.0	59.2	5.3
HK:HAA	12.1	45.3	5.5	8.2	26.3	2.1
HK_{KYNU} = HK:(AA + HAA)	11.5	52.3	6.0	9.2	33.1	3.0
HKr = HK:(KA + XA + AA + HAA)	12.8	60.8	7.8	11.6	61.7	7.2
Women						
HK	7.3	24.2	1.8	4.9	11.5	0.6
HK:XA	8.0	25.5	2.0	12.1	34.9	4.2
HK_{KAT} = HK:(KA + XA)	16.2	67.6	10.9	16.5	62.2	10.3
HK:HAA	18.4	57.5	10.6	11.7	29.9	3.5
HK_{KYNU} = HK:(AA + HAA)	19.3	66.6	12.9	14.4	38.5	5.5
HKr = HK:(KA + XA + AA + HAA)	21.9	73.1	16.0	19.0	58.6	11.1

[1]Performance characteristics were calculated based on multiple linear regression and the "lmg" algorithm as implemented in the "relaimpo" package in R, as further described in "Statistical methods." (The main output from the method is shown in Figure 2.) AA, anthranilic acid; HAA, 3′hydroxyanthranilic acid; HK, 3′hydroxykynurenine; HKr, HK ratio; HUSK, Hordaland Health Study; KA, kynurenic acid; KAT, kynurenine transaminase; KYNU, kynureninase; WECAC, Western Norway Coronary Angiography Cohort; XA, xanthurenic acid.

GAM plots of the change in HKr against change in PLP in the nontreated and pyridoxine-treated groups. Standardized linear regression coefficients for the associations in the nontreated and treated groups were −0.34 and −0.48, respectively. Corresponding associations were −0.33 and −0.44 for HK:XA and −0.11 and −0.33 for HK. The mean overall reductions in HKr, HK:XA, and HK in the vitamin B-6–treated groups were 39%, 31%, and 34%, respectively. The aforementioned findings were not altered by adjustment for the folic acid + cobalamin treatment arm.

PLP and HKr by age and sex

We found a steady and, apparently, slightly accelerating decline in vitamin B-6 status with age among WECAC men as indicated by both PLP and HKr. For women, B-6 status appeared to improve until age 55 y and then declined at an increasing rate at age >55 y. Again, the PLP and HKr findings closely mimicked each other (**Figure 5**).

Discussion

Principal findings

In the present study we evaluated both circulating concentrations and ratios of Kyns as potential functional markers of vitamin B-6 status in 1 community-based and 1 clinical cohort. Among the panel of candidate markers, the best performance characteristics were found for the ratio of HK to (KA + XA + HAA + AA), abbreviated HKr. Compared with Kyn-based markers proposed previously (13) and additional markers included in this study, HKr demonstrated stronger associations with PLP, in both cross-sectional and longitudinal analyses, and also considerably increased specificity for PLP. The findings were consistent across cohorts and subgroups and featured a 2-segmented dose–response curve with a cutoff close to 20 nmol/L, a threshold suggested to indicate B-6 deficiency (29, 30).

Possible mechanisms

Previously, we reported the characteristics of the 2 substrate product ratios HK:XA and HK:HAA and the closely related HK:KA and HK:AA within the WENBIT cohort (13). The rationale for using substrate:product pairs was discussed previously (13) and a more theoretical basis may be found in metabolic control theory (31). Briefly, by taking ratios, the influence of confounders common to the nominator and denominator would tend to be attenuated, whereas information related to enzyme dependency, in this case the intracellular availability of PLP, would be amplified. Interestingly, the best overall marker in the current study was a construct made of HK in the nominator and the sum of all 4 Kyns downstream of the 2 PLP-dependent enzymes KAT and KYNU in the denominator. To gain a better understanding we also evaluated ratios limited to KAT, i.e., HK:(KA + XA), and KYNU, i.e., HK:(AA + HAA), and observed characteristics intermediate to those of the corresponding simple ratio (e.g., HK:HAA) and the full HKr.

Closer examination of the results in Table 3 showed that a main benefit of using sums of downstream Kyns, e.g., KA + XA, in the denominator was an increase in specificity. Further, the main benefit of using the full HKr over HK_{KAT} and HK_{KYNU} was greater consistency in performance across cohorts and genders. The ratios Kyn:KA and Kyn:AA were only weakly related to PLP. The likely reason is that Kyn is readily converted to HK by FAD-dependent kynurenine monooxygenase (KMO) and thus does not accumulate as PLP becomes limiting. Riboflavin status has been shown to affect the activity of KMO (32), but did not materially affect the relation between PLP and B-6 markers in the present study. The mean concentrations of KA, XA, HAA, and AA differed by ≤3-fold, but correlation analysis showed that variation in their sum (as used in the denominator of HKr) was not overly dominated by any one of the individual Kyns. Notably, a construct using the product of downstream Kyns in the denominator was inferior to HKr. Similarly, replacing the downstream Kyns with the sum or the product of their standardized equivalents did not improve overall performance characteristics. A likely reason for the utility of the plain sum of KA, XA, AA, and HAA in HKr may be that they all share the same source: Kyn.

Reproducibility of findings

In our previous report on substrate:product ratios, we concluded that HK:XA had slightly better characteristics than HK:HAA as a potential functional marker of vitamin B-6 status (13). Using a more stringent (quantitative) analysis based on relative importance regression, we confirmed this finding in the larger WECAC cohort. In HUSK, however, the performance of HK:XA was clearly inferior to that of HK:HAA. The reason for this discrepancy is not clear. The performance of HKr was, by comparison, consistent. Conceivably, this could be explained by HKr capturing information from both enzymes, which might have a stabilizing effect on performance across cohorts and subgroups.

HKr- and PLP-based cutoffs for overt and marginal vitamin B-6 deficiency

The HKr index demonstrated a markedly increased sensitivity to changes in PLP concentration at PLP concentrations below ∼19 nmol/L in both the HUSK and WECAC cohorts. This result may be regarded as supportive for the concept of HKr as a functional marker of vitamin B-6 status, but, conversely, it can also be viewed as novel and direct metabolic support for a cutoff of 20 nmol/L for vitamin B-6 deficiency. Several investigators have studied a related concept of marginal deficiency defined as PLP concentrations in the interval 20–30 nmol/L (33). In the GAM analyses there was some support for a segment in the interval 20–40 nmol PLP/L where the association with HKr was intermediate. The HKr decreased further beyond 40 nmol/L PLP; thus, it would be hard to use the present data to argue for a specific threshold for suboptimal vitamin B-6 status. Although the data only offer limited support, they certainly do not conflict with a concept of marginal vitamin B-6 deficiency in an interval stretching from 20 to 30 nmol PLP/L or even above.

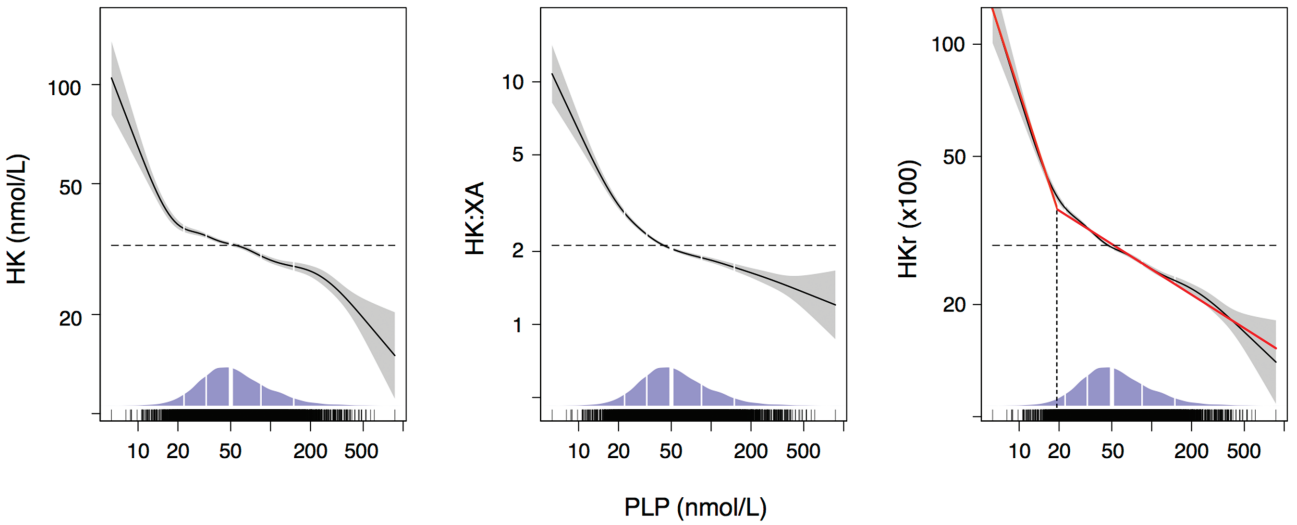

FIGURE 3 Association of HK, HK:XA, and HKr with PLP in the Hordaland Health Study by GAMs. Gray-shaded areas indicate the 95% CI. A density plot for the distribution of PLP is included in each panel with white lines indicating the 5th, 20th, 50th, 80th, and 95th percentiles. The horizontal dotted line marks the adjusted mean concentration of the vitamin B-6 marker. For the HKr association, 2 segments, calculated by segmented regression, are overlaid (red color) on the GAM curve, and a significant breakpoint (95% CI) at 19.4 (18.1, 20.7) nmol/L is indicated by the vertical dotted line. GAM, generalized additive model; HK, 3′hydroxykynurenine; HKr, HK ratio; PLP, pyridoxal 5′-phosphate; XA, xanthurenic acid.

HKr and differences according to age and sex

HKr was markedly higher in women than in men in both HUSK and WECAC (13% and 18% higher, respectively, age-adjusted), whereas corresponding values for PLP were 4% higher and 5% lower. Notably, both KAT and KYNU have been found to be inhibited by estrogen (34), which could explain the lower concentrations of KA, XA, and HAA, and therefore higher HKr, in women. Declining estrogen concentrations (35) could also potentially explain the downward trend in HKr until ∼55 y. Notably, however, high estrogen, e.g., from oral contraceptives, or around the time of ovulation, is associated with low PLP (30). Thus, the similarity of the PLP– and HKr–age association curves suggests that the age-related differences in HKr are mediated through changes in PLP rather than resulting from direct effects of estrogen on KAT and KYNU. In men, and in women older than 55 y, vitamin B-6 status decreased according to both indicators. The rate of decline corresponded well with a previously published value of 4 nmol/decade (36) and with other reports (37). Possible explanations could include increased inflammation and/or age-related differences in nutrition (3, 37).

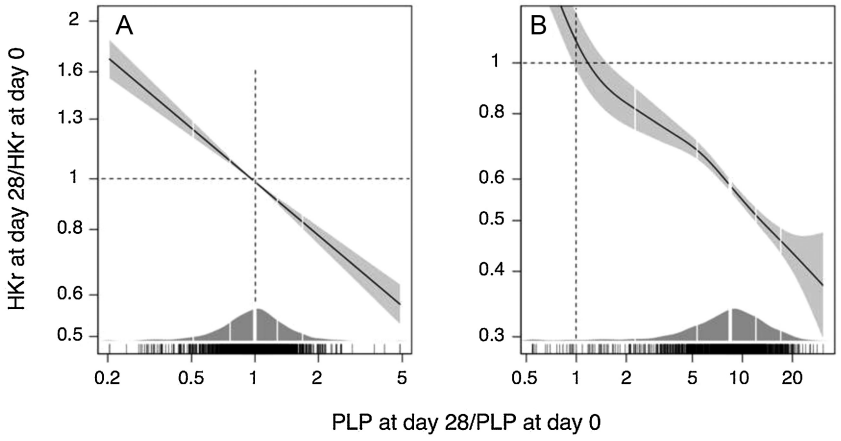

FIGURE 4 Change in HKr compared with change in PLP by generalized additive models. Change is defined as the concentration of the vitamin B-6 marker 28 d into the study (Western Norway B-Vitamin Intervention Trial) divided by the concentration at baseline (day 0). (A) Nontreated groups (n = 1130). (B) Groups treated with a daily oral dose of 40 mg pyridoxine (n = 1138). Gray-shaded areas denote the 95% CI. The distribution of PLP at day 28/PLP at day 0 is shown at the bottom of each panel with white lines indicating the 5th, 20th, 50th, 80th, and 95th percentiles. Horizontal and vertical dotted lines indicate where no change from baseline to day 28 is found (ratio = 1). HKr, HK ratio; PLP, pyridoxal 5′-phosphate.

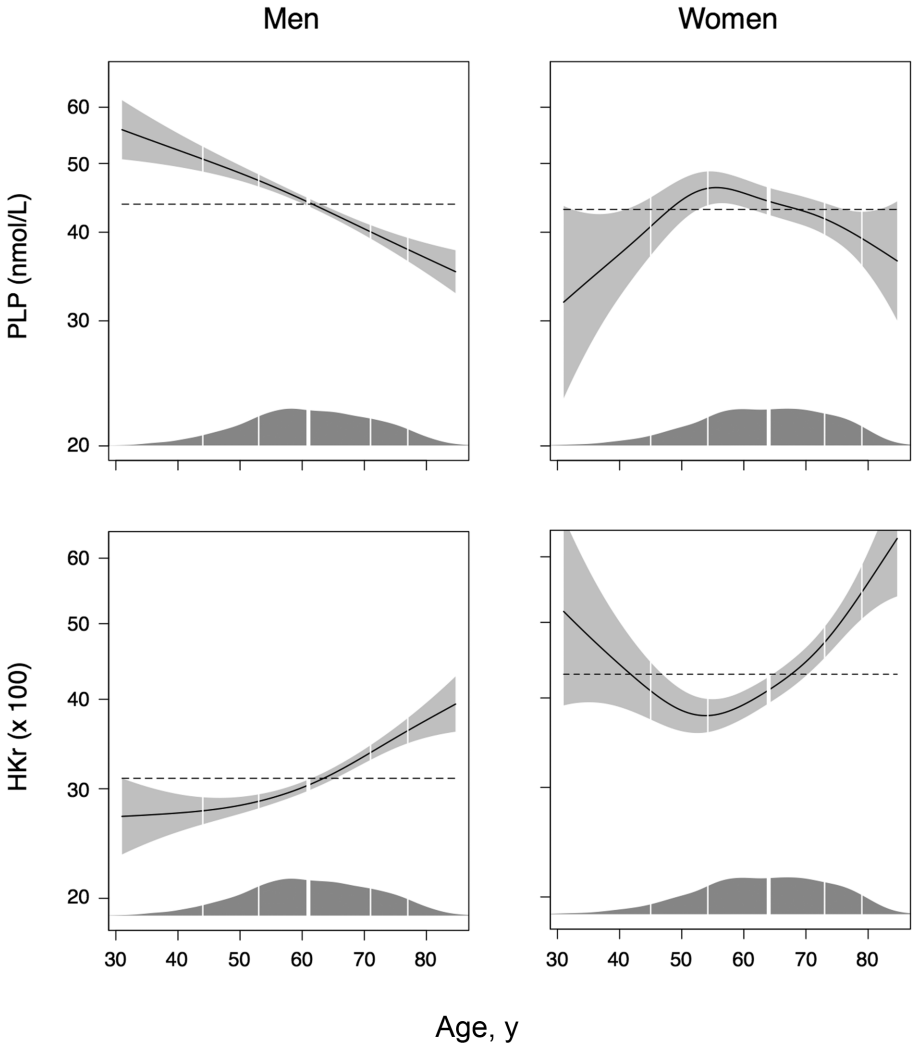

FIGURE 5 The association of HKr with age in the Western Norway Coronary Angiography Cohort by generalized additive models. Gray-shaded areas denote the 95% CI. The age distribution is shown at the bottom of each panel with white lines indicating the 5th, 20th, 50th, 80th, and 95th percentiles. The horizontal dotted line in each panel indicates the adjusted mean concentration of the vitamin B-6 marker. HKr, HK ratio; PLP, pyridoxal 5′-phosphate.

Strengths and limitations

The main strengths of the study included the use of an established MS-based assay that quantifies Trp, all the Kyns, and PLP in a single run. We were able to use data from 2 large cohorts with notable differences in characteristics to assess reproducibility and consistency. Furthermore, data from WENBIT participants allowed us to evaluate both longitudinal aspects and responses to intervention with vitamin B-6 (pyridoxin). The main limitation was the lack of a third, independent, marker of vitamin B-6 status. We could only evaluate the Kyn-based markers against plasma PLP.

Conclusions

In this article we have described an in-depth exploration of circulating Kyns as functional markers of vitamin B-6 status. The marker with best performance and overall characteristics was a construct, HKr, which included 5 of the 6 metabolites immediately up- and downstream of the 2 PLP-dependent enzymes in the Kyn pathway. The results for HKr were reproducible across cohorts and subgroups, and its appropriateness was further corroborated by highly sex-specific age-associations indicated by both PLP and HKr.

Many of the Kyns measured in this study have neuromodulatory and/or immunological effects and have been linked to various pathologies including psychiatric disorders, cognitive decline, cancer, and cardiovascular disease (38, 39). Because low vitamin B-6 status has been found for many of the same conditions (10, 11), it should be of great value to jointly investigate Kyns and vitamin B-6 status in future studies of clinical outcomes.

The authors' responsibilities were as follows—AU, ON, GT, and PMU: designed the research; ØM, AM, and KM: conducted the research; AU: performed the statistical analysis, wrote the paper, and had primary responsibility for the final content; and all authors: read and approved the final manuscript. None of the authors reported a conflict of interest related to the study.

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