

## Vitamins B<sub>2</sub> and B<sub>6</sub> as determinants of kynurenines and related markers of interferon- $\gamma$ -mediated immune activation in the community-based Hordaland Health Study

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### Abstract

Vitamins B<sub>2</sub> and B<sub>6</sub> are cofactors in the kynurenine pathway. Many of the kynurenines are neuroactive compounds with immunomodulatory effects. In the present study, we aimed to investigate plasma concentrations of vitamins B<sub>2</sub> and B<sub>6</sub> as determinants of kynurenines and two markers of interferon- $\gamma$ -mediated immune activation (kynurenine:tryptophan ratio (KTR) and neopterin). We measured the concentrations of vitamins B<sub>2</sub> and B<sub>6</sub> vitamers, neopterin, tryptophan and six kynurenines (i.e. kynurenine, anthranilic acid, kynurenic acid, 3-hydroxykynurenine, 3-hydroxyanthranilic acid and xanthurenic acid) in plasma from 7051 individuals. Dietary intake of vitamins B<sub>2</sub> and B<sub>6</sub> was assessed using a validated FFQ. Associations were investigated using partial Spearman's correlations, generalised additive models, and segmented or multiple linear regression. The B<sub>2</sub> vitamer, riboflavin, was positively associated with 3-hydroxyanthranilic acid and xanthurenic acid, with correlation coefficients, as obtained by segmented regression, of 0.20 (95% CI 0.16, 0.23) and 0.24 (95% CI 0.19, 0.28), at riboflavin concentrations below the median value (13.0 nmol/l). The vitamin B<sub>6</sub> vitamer, pyridoxal 5'-phosphate (PLP), was positively associated with most kynurenines at PLP concentrations < 39.3–47.0 nmol/l, and inversely associated with 3-hydroxykynurenine with the association being more prominent at PLP concentrations < 18.9 nmol/l. Riboflavin and PLP were associated with xanthurenic acid only at relatively low, but normal concentrations of both vitamers. Lastly, PLP was negatively correlated with neopterin and KTR. These results demonstrate the significant and complex determination of kynurenine metabolism by vitamin status. Future studies on B-vitamins and kynurenines in relation to chronic diseases should therefore integrate data on relevant biomarkers related to B-vitamins status and tryptophan metabolism.

**Key words:** Tryptophan: Riboflavin: Pyridoxal 5'-phosphate: Inflammation

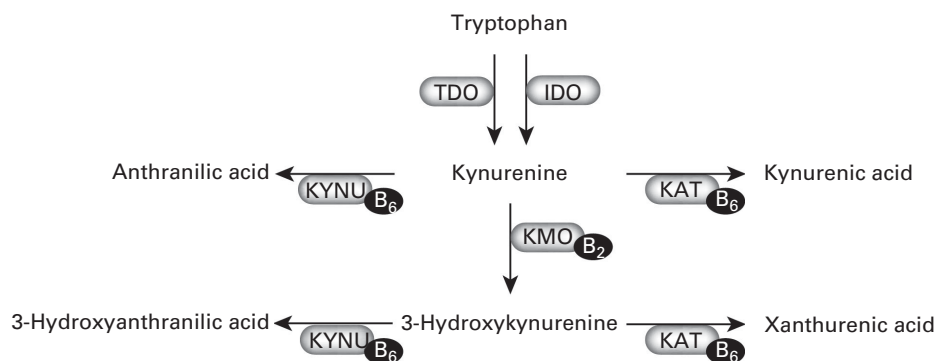
Tryptophan is mainly catabolised through the kynurenine pathway, which accounts for more than 90% of tryptophan degradation<sup>(1)</sup>. Several metabolites of this pathway, collectively labelled kynurenines, have distinct immune-regulatory<sup>(2,3)</sup> and/or neuroactive properties<sup>(4,5)</sup>. Kynurenines may be involved in a wide range of clinical conditions, including neurodegenerative disorders<sup>(6)</sup>, autoimmune diseases<sup>(4)</sup>, infection<sup>(4)</sup>, cancer<sup>(4)</sup> and the metabolic syndrome<sup>(7)</sup>. The flux of tryptophan catabolism through the kynurenine pathway, reflected by the kynurenine:tryptophan ratio (KTR), is increased during

inflammation, primarily in response to interferon- $\gamma$ , which also stimulates the production of the pteridine neopterin<sup>(4)</sup>. Thus, KTR and neopterin are markers of interferon- $\gamma$ -mediated cellular immune activation and, as such, associated with chronic diseases involving inflammatory processes<sup>(4,8)</sup>.

Factors other than inflammation may also influence tryptophan catabolism through the kynurenine pathway. Vitamins B<sub>2</sub> and B<sub>6</sub>, in the form of FAD and pyridoxal 5'-phosphate (PLP), respectively, are cofactors for enzymes in this pathway. The conversion of kynurenine to 3-hydroxykynurenine by kynurenine

**Abbreviations:** GAM, generalised additive models; KTR, kynurenine:tryptophan ratio; PLP, pyridoxal 5'-phosphate.

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**Fig. 1.** The kynurenine pathway. Tryptophan is converted to kynurenine by either indoleamine 2,3-dioxygenase (IDO) or tryptophan 2,3-dioxygenase (TDO). Kynurenine is further metabolised to anthranilic acid by kynureninase (KYNU) or kynurenic acid by kynurenine aminotransferases (KAT), with B<sub>6</sub> in the form of pyridoxal 5'-phosphate (PLP) acting as a coenzyme, or 3-hydroxykynurenine by kynurenine mono-oxygenase (KMO), with vitamin B<sub>2</sub> in the form of FAD acting as a coenzyme. In turn, 3-hydroxykynurenine is converted to either 3-hydroxyanthranilic acid by KYNU or xanthurenic acid by KAT, again with PLP acting as a coenzyme. This schematic is a modification of a previously published figure<sup>(26)</sup>.

mono-oxygenase requires FAD, whereas PLP is a cofactor for the conversion of kynurenine to anthranilic acid, and 3-hydroxykynurenine to 3-hydroxyanthranilic acid, both catalysed by kynureninase, as well as for the conversion of kynurenine to kynurenic acid, and 3-hydroxykynurenine to xanthurenic acid, catalysed by kynurenine aminotransferase<sup>(9)</sup> (Fig. 1).

Early studies have described altered excretion of kynurenes in the urine during vitamin B<sub>2</sub><sup>(10,11)</sup> or B<sub>6</sub> deficiency<sup>(12)</sup>. Increased excretion of xanthurenic acid in the urine following a tryptophan loading test has been used as a measure of vitamin B<sub>6</sub> deficiency<sup>(13)</sup>. More recently, a study in patients with stable angina pectoris has shown that plasma PLP was associated with several kynurenes, and suggested that plasma 3-hydroxykynurenine<sup>(14)</sup> as well as substrate product ratios including kynurenes are potential metabolic markers of functional B<sub>6</sub> status<sup>(15)</sup>.

The most commonly used marker of vitamin B<sub>6</sub> status is circulating PLP, but two other forms of vitamin B<sub>6</sub> are also present in plasma<sup>(16)</sup>, i.e. pyridoxal, which can be converted to PLP and vice versa, and 4-pyridoxic acid, the predominant catabolic product of vitamin B<sub>6</sub><sup>(17)</sup>. Vitamin B<sub>2</sub> is present in plasma as riboflavin and the coenzyme forms FAD and FMN. All forms are interconvertible<sup>(18)</sup>, and plasma riboflavin may serve as an indicator of vitamin B<sub>2</sub> status in population studies<sup>(19)</sup>.

Despite the key roles of vitamins B<sub>2</sub> and B<sub>6</sub> in the kynurenine pathway, data on the relationship of vitamin B<sub>2</sub> and B<sub>6</sub> status with circulating kynurenes in the general population are lacking. Therefore, in the present study, we investigated the associations of plasma concentrations and dietary intake of vitamins B<sub>2</sub> and B<sub>6</sub> with six kynurenes, KTR and neopterin, in 7051 individuals who participated in a large community-based cohort study.

## Methods

### Study population

The source population included residents of the city of Bergen, Norway, or neighbouring municipalities, who participated in the Hordaland Health Study during 1997–1999 and

were born in 1925–1927 or 1950–1951 (*n* 9187). The study population consisted of 7051 participants (attendance rate 77%) in two distinct age groups: 3727 participants aged 46–47 years and 3324 participants aged 70–72 years. The study protocol of the Hordaland Health Study was approved by the Western Norway Regional Committee for Medical and Health Research Ethics. All participants gave written informed consent<sup>(20)</sup>.

### Blood collection and biochemical analyses

Blood samples were collected into tubes containing EDTA and kept on ice within 15–30 min. The samples were centrifuged within 3 h, and EDTA plasma was stored at  $-80^{\circ}\text{C}$  until analysis. Plasma concentrations of vitamin B<sub>2</sub> species (riboflavin and FMN) and B<sub>6</sub> species (PLP, pyridoxal and 4-pyridoxic acid) as well as neopterin, tryptophan, kynurenine, anthranilic acid, kynurenic acid, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, xanthurenic acid and cotinine, an established marker of recent nicotine exposure<sup>(21)</sup>, were measured using a high-throughput liquid chromatography–tandem MS assay<sup>(22)</sup>. KTR was calculated by dividing the plasma concentration of kynurenine by the concentration of tryptophan and subsequently multiplying by 1000. Plasma creatinine was measured by HPLC–MS/MS<sup>(23)</sup>, and was used for calculating estimated glomerular filtration rate, which is a measure of renal function, using the Chronic Kidney Disease Epidemiology Collaboration equation<sup>(24)</sup>. All biochemical analyses were performed in the laboratory of Bevital AS (<http://www.bevital.no>). CV were between 2.0 and 16.9%<sup>(22,23)</sup>.

### Data collection on health and diet

Weight and height were measured and BMI was calculated as weight/height<sup>2</sup> (kg/m<sup>2</sup>). Self-reported information was available on the prevalence of chronic diseases such as myocardial infarction, stroke, diabetes, as well as on smoking status (current, former or never). We combined the questionnaire data on smoking with plasma cotinine concentration to define never smokers (no self-reported previous smoking

and plasma cotinine concentration  $\leq 85$  nmol/l), former smokers (self-reported previous smoking and plasma cotinine concentration  $\leq 85$  nmol/l) and current smokers (plasma cotinine concentration  $> 85$  nmol/l)<sup>(21)</sup>. Dietary intake of vitamins B<sub>2</sub> and B<sub>6</sub> was calculated using a validated FFQ, which was a modified version of a questionnaire developed at the Department of Nutrition, University of Oslo<sup>(25)</sup>. Dietary data were available for 6117 individuals.

### Statistical analyses

Population characteristics are presented as medians and 5th–95th percentiles for continuous variables, and as counts (proportions) for discrete variables. Differences between groups were tested using the Mann–Whitney *U* test for continuous variables (as most variables were non-normally distributed) and by the  $\chi^2$  test for discrete variables. Partial Spearman's correlations, adjusted for age, sex and renal function (estimated glomerular filtration rate), were used to investigate the associations of vitamin B<sub>2</sub> and B<sub>6</sub> species with neopterin, KTR, tryptophan and kynurenines. We additionally adjusted correlations for energy intake when investigating the associations of dietary intake of vitamins B<sub>2</sub> and B<sub>6</sub> with neopterin, KTR, tryptophan and kynurenines. Dietary data were available for 6117 individuals, of which we excluded those with implausible reported energy intake (127 women:  $> 15\,000$  or  $< 3000$  kJ/d and seventy-one men:  $> 17\,500$  or  $< 3300$  kJ/d), yielding a sample of 5919 participants for analysis regarding dietary intake. We identified non-linear associations by generalised additive models (GAM), with both outcome and exposure variables being log-transformed (natural logarithm). In cases where a non-linear relationship was suggested by the GAM curves, segmented regression assuming a two-segmented model with a single breakpoint was used, again with log-transformed data. After accounting for missing data by list-wise deletion, 6931 participants with complete data were available for analysis. Since segmented regression can be influenced by outliers, we excluded participants with plasma riboflavin or PLP concentration above the 99th percentile ( $n = 70$ ). This resulted in a final sample size of 6861 participants with complete data for analysis. The 99th percentile corresponded to 156.1 nmol/l for riboflavin and 265.7 nmol/l for PLP. In addition, ten participants with extreme values of 3-hydroxykynurenine ( $> 185$  nmol/l) were excluded from the analysis for this outcome ( $n = 6851$ ). For consistency, the same exclusion criteria were applied to the two-dimensional GAM curves. We additionally checked for interactions between riboflavin and PLP as predictors of kynurenines by adding a product term in regression models and visualising with three-dimensional GAM plots. All models were adjusted for age, sex and renal function (estimated glomerular filtration rate), as those factors are strong determinants of kynurenines<sup>(26)</sup>. Statistical analyses were performed using SAS 9.2 (SAS Institute, Inc.). We used R (version 2.14.1 for Windows)<sup>(27)</sup> for construction of two- and three-dimensional GAM curves (package 'mgcv'), and for segmented regression analysis (package 'segmented').

## Results

### Characteristics of the study population

The study population consisted of 3727 participants aged 46–47 years and 3324 participants aged 70–72 years. Approximately 45% of the participants were men. For both sexes, most kynurenines, KTR and neopterin were higher in the elderly than in middle-aged participants. The concentrations of vitamin B<sub>2</sub> and B<sub>6</sub> species also varied somewhat according to age and sex. Plasma concentrations of riboflavin were lower in men than in women, and higher in the older group. Plasma PLP concentration was highest in middle-aged men and older women. Pyridoxal and 4-pyridoxic acid concentrations were highest in the older group (Table 1).

### Correlations between vitamins

The two vitamin B<sub>2</sub> species, riboflavin and FMN, were positively correlated ( $r = 0.52$ ), as were the three vitamin B<sub>6</sub> species ( $r = 0.68$ – $0.79$ ). Riboflavin and, to a lesser extent, FMN were associated with vitamin B<sub>6</sub> species with correlations ranging from 0.23 to 0.48. Riboflavin and FMN were positively correlated with vitamin B<sub>2</sub> intake including ( $r = 0.22$  and 0.13, respectively) and excluding supplement use ( $r = 0.14$  and 0.09, respectively). B<sub>6</sub> species were positively correlated with B<sub>6</sub> intake with  $r$  ranging from 0.24 to 0.27 for intake including supplements and from 0.18 to 0.20 for intake excluding supplements. Vitamin B<sub>2</sub> and B<sub>6</sub> intakes were positively correlated ( $r = 0.38$ ). All aforementioned correlations were statistically significant ( $P < 0.0001$ ).

### Kynurenines, kynurenine:tryptophan ratio and neopterin according to B-vitamin status

The associations of kynurenines, KTR and neopterin with dietary intake and plasma concentrations of vitamins B<sub>2</sub> and B<sub>6</sub> were evaluated by partial Spearman's correlation adjusted for age, sex and renal function (estimated glomerular filtration rate). When dietary B-vitamins were considered, we additionally adjusted for energy intake. The results are summarised in Table 2.

The correlations of kynurenines with vitamin B<sub>2</sub> status were generally weak or null, except for 3-hydroxyanthranilic acid and xanthurenic acid, which showed a moderate and positive association with riboflavin concentration ( $r = 0.19$  and 0.13, respectively) and vitamin B<sub>2</sub> intake ( $r = 0.13$  and 0.16, respectively) (Table 2). These associations were non-linear (Fig. 2), and therefore they were further investigated by segmented regression (Table 3). Riboflavin was positively associated with 3-hydroxyanthranilic acid ( $\beta$ -coefficient 0.20, 95% CI 0.16, 0.23) and xanthurenic acid ( $\beta$ -coefficient 0.24, 95% CI 0.19, 0.28) at riboflavin concentrations below 12.2 and 13.5 nmol/l, respectively, which are close to the median riboflavin concentration in the study population (13 nmol/l). At higher concentrations, the dose–response curves for riboflavin and these kynurenines reached a plateau (Fig. 2). Additional adjustment for PLP did not materially change the associations between riboflavin and the kynurenines (data

**Table 1.** Characteristics of the participants in the Hordaland Health Study stratified by age and sex† (Number of participants and percentages; median values and 5th (P5)–95th (P95) percentiles)

Variables§	46–47 years				70–72 years			
	Women (n 2064)		Men (n 1663)		Women (n 1854)		Men (n 1470)	
	Median	P5–P95	Median	P5–P95	Median	P5–P95	Median	P5–P95
Supplement use								
n	343		200*		323		185*	
%	20		15		21		14	
Disease¶								
n	25		53		259†		354†	
%	1.2		3.2		14		24	
Current smoking								
n	746		619		301†		292†	
%	37		38		17		20	
BMI (kg/m <sup>2</sup> )	24.1	19.9–32.6	25.9*	21.4–31.8	26	19.6–34.1	25.8†	21.0–31.6
Creatinine (μmol/l)	72	60–88	86*	71–154	76†	60–100	92†*	73–123
eGFR (ml/min per 1.73 m <sup>2</sup> )	86	62–96	88*	70–109	68†	49–88	75†*	54–98
Total B <sub>2</sub> intake** (mg/d)	1.55	0.79–3.02	1.93*	1.07–3.48	1.38†	0.69–3.05	1.60†*	0.86–3.03
Dietary B <sub>2</sub> intake†† (mg/d)	1.44	0.77–2.44	1.85*	1.05–3.07	1.32†	0.68–2.21	1.54†*	0.83–2.58
Riboflavin (nmol/l)	12.4	4.9–61.3	11.4*	4.8–38.5	16.2†	5.3–91.5	12.9	4.6–62.1
FMN (nmol/l)	13.1	7.1–27.0	12.8	7.3–25.8	13.0	7.0–24.4	11.4†*	6.4–23.7
Total B <sub>6</sub> intake** (mg/d)	1.36	0.75–2.66	1.67	0.97–2.99	1.16†	0.57–2.8	1.44	0.76–2.82
Dietary B <sub>6</sub> intake†† (mg/d)	1.31	0.74–2.09	1.63	0.96–2.62	1.14†	0.57–1.96	1.41†*	0.76–2.29
PLP (nmol/l)	47.3	21.7–147	55.6*	27.1–133	52.6†	21.6–185	44.6†*	20.7–127
PL (nmol/l)	11.2	6.2–39.2	11.6	6.8–26.9	12.7†	6.5–51.5	12.2†	6.7–37.8
PA (nmol/l)	19.7	10.2–95.1	20.3	11.6–60.3	25.9†	12.6–162	25.7†	14.0–113
Trp (μmol/l)	65.9	46.4–91.9	72.5*	53.4–98.1	62.5†	42.5–89.8	67.9†*	47.9–95.7
Kyn (μmol/l)	1.30	0.89–2.02	1.47*	1.02–2.15	1.65†	1.05–2.52	1.79†*	1.20–2.47
AA (nmol/l)	12.7	7.6–22.1	12.9	8.0–22.2	15.9†	9.7–28.1	16.8†*	10.0–29.1
KA (nmol/l)	39.1	22.9–68.4	45.1*	26.2–79.1	48.1†	26.8–86.8	54.5†*	29.9–98.5
HK (nmol/l)	30.3	17.6–53.6	28.7*	17.3–46.7	35.3†	20.5–66.1	36.2†*	21.8–64.6
HAA (nmol/l)	31.1	17.4–59.4	34.6*	18.9–60.8	32.7†	17.6–61.0	36.0†*	19.3–68.2
XA (nmol/l)	15.3	6.4–34.0	17.1*	7.9–33.6	14.0†	5.6–30.6	16.6*	7.1–34.7
Neopterin (nmol/l)	7.1	4.8–11.3	6.7*	4.6–10.4	8.7†	5.7–15.6	8.6†	5.8–15.8
KTR (nmol/μmol)	19.9	13.5–31.3	20.1	13.2–31.1	26.3†	16.3–43.5	26.1†	16.5–44.2

eGFR, estimated glomerular filtration rate; PLP, pyridoxal 5'-phosphate; PL, pyridoxal; PA, 4-pyridoxic acid; Kyn, kynurenine; AA, anthranilic acid; KA, kynurenic acid; HK, 3-hydroxykynurenine; HAA, 3-hydroxyanthranilic acid; XA, xanthurenic acid; KTR, kynurenine:tryptophan ratio.

\* Median value was significantly different from that of women ( $P < 0.01$ ).

† Median value was significantly different from that of the 46- to 47-year age group ( $P < 0.01$ ).

‡ Differences between the groups were investigated using the Mann–Whitney  $U$  test for continuous variables and by the  $\chi^2$  test for discrete variables.

§ Numbers of observations available differed between the variables: BMI ( $n$  7031); creatinine ( $n$  7021); eGFR ( $n$  7021); riboflavin, FMN, PLP, PA ( $n$  6956); neopterin, Trp, KA ( $n$  6956); KTR, Kyn, AA ( $n$  6934); HK ( $n$  6953); HAA ( $n$  6954); XA ( $n$  6955). Some characteristics included in this table have been published previously<sup>(26)</sup>. Creatinine, vitamin species, neopterin and metabolites were measured in plasma.

|| Use of multivitamin or B-vitamin supplements during the whole year, only including participants with reliable dietary intake information, defined as 3000–15 000 kJ/d for women and 3300–17 500 kJ/d for men ( $n$  5919).

¶ Defined as self-reported history of either myocardial infarction, stroke or diabetes.

\*\* Including intake from supplements and foods, in participants with reliable dietary intake ( $n$  5919).

†† Including intake from foods only, in participants with reliable dietary intake ( $n$  5919).

not shown). Plasma concentrations of vitamin B<sub>2</sub> species were not associated with KTR or neopterin.

PLP was positively correlated with tryptophan and most kynurenines ( $r$  0.08–0.19), except for the null association with kynurenine and the inverse correlation with 3-hydroxykynurenine ( $r$  –0.26). Additionally, PLP was inversely correlated with both the inflammatory markers KTR ( $r$  –0.17) and neopterin ( $r$  –0.14). All aforementioned correlations were statistically significant ( $P < 0.001$ ). Similar, but weaker, correlations were observed for pyridoxal and 4-pyridoxic acid. Intake of vitamin B<sub>6</sub> showed weak or null associations with kynurenines or inflammatory markers (Table 2).

Several of the associations of PLP with kynurenines were non-linear. PLP was positively associated with tryptophan, kynurenic acid, 3-hydroxyanthranilic acid and xanthurenic acid at PLP concentrations  $< 39.3$  nmol/l (40th percentile),

reaching a plateau at higher PLP concentrations. PLP was strongly inversely associated with 3-hydroxykynurenine, in particular, at PLP concentrations  $< 18.9$  nmol/l (3rd percentile;  $\beta$ -coefficients –0.59, 95% CI –0.85, –0.33; Table 3 and Fig. 2). Additional adjustment for riboflavin did not affect the associations of PLP with tryptophan and kynurenines (data not shown). Results were similar across the tertiles of inflammatory markers (data not shown).

#### Interaction effects of vitamins B<sub>2</sub> and B<sub>6</sub> as predictors of kynurenines

We also investigated possible interactions between vitamins B<sub>2</sub> (riboflavin) and B<sub>6</sub> (PLP) as predictors of kynurenines. The positive associations of both riboflavin and PLP with xanthurenic acid were essentially confined to participants

**Table 2.** Correlations of vitamins B<sub>2</sub> and B<sub>6</sub> with kynurenines (Kyn) and markers of interferon- $\gamma$ -mediated immune activation for the participants in the Hordaland Health Study†

	Trp	Kyn	AA	KA	HK	HAA	XA	Neopterin	KTR
Riboflavin	0.10***	0.06***	0.11***	0.13***	-0.01	0.19***	0.13***	0.03	-0.02
FMN	-0.05***	-0.10***	0.10***	0.12***	-0.08***	0.06***	0.05***	0.01	-0.05***
Total B <sub>2</sub> intake‡	0.06***	0.01	0.03	0.05***	-0.03	0.10***	0.08***	0.00	-0.04
PLP	0.19***	-0.03	0.08***	0.13***	-0.26***	0.16***	0.11***	-0.14***	-0.17***
PL	0.13***	0	0.14***	0.11***	-0.22***	0.13***	0.07***	-0.07***	-0.09***
PA	0.07***	0.08***	0.13***	0.20***	-0.13***	0.16***	0.12***	0.05***	0.02
Total B <sub>6</sub> intake‡	0.06***	-0.01	0.01	0.07***	-0.06***	0.08***	0.05***	-0.08***	-0.05***

AA, anthranilic acid; KA, kynurenic acid; HK, 3-hydroxykynurenine; HAA, 3-hydroxyanthranilic acid; XA, xanthurenic acid; KTR, kynurenine:tryptophan ratio; PLP, pyridoxal 5'-phosphate; PL, pyridoxal; PA, 4-pyridoxic acid.

\*\*\*  $P < 0.001$  (Spearman's correlations, adjusted for age, sex and renal function ( $n = 6921$  for HK and  $n = 6931$  for all other metabolites)).

† Creatinine, vitamin species, neopterin and metabolites were measured in plasma.

‡ Total intake from foods and supplements. Correlations were additionally adjusted for energy intake ( $n = 5813$  for HK and  $n = 5821$  for all other metabolites). Analysis restricted to participants with plausible reports of energy intake, defined as 3000–15 000 kJ/d for women and 3300–17 500 kJ/d for men.

with low (below the median) concentrations of the other vitamin (Fig. 3). Both riboflavin and PLP were positively associated with 3-hydroxyanthranilic acid, throughout the distribution of the other vitamin, and no interaction between vitamins B<sub>2</sub> and B<sub>6</sub> was observed for 3-hydroxykynurenine, which was only dependent on PLP concentrations (data not shown).

## Discussion

### Principal findings

Vitamins B<sub>2</sub> and B<sub>6</sub> are cofactors in the kynurenine pathway, which includes neuroactive metabolites with immunomodulatory effects<sup>(4)</sup>. We investigated vitamins B<sub>2</sub> and B<sub>6</sub> as determinants of kynurenines, and the related markers of interferon- $\gamma$ -mediated immune activation, KTR and neopterin, in 7051 healthy participants. Riboflavin at concentrations below the population median was positively associated with 3-hydroxyanthranilic acid and xanthurenic acid. In addition, riboflavin and PLP were interactive determinants of xanthurenic acid. PLP was positively associated with all kynurenines formed by PLP-dependent enzymes, kynureninase and kynurenine aminotransferases, but inversely associated with 3-hydroxykynurenine, which is metabolised by PLP-dependent enzymes. Similar observations have previously been made in cardiovascular patients<sup>(15)</sup>.

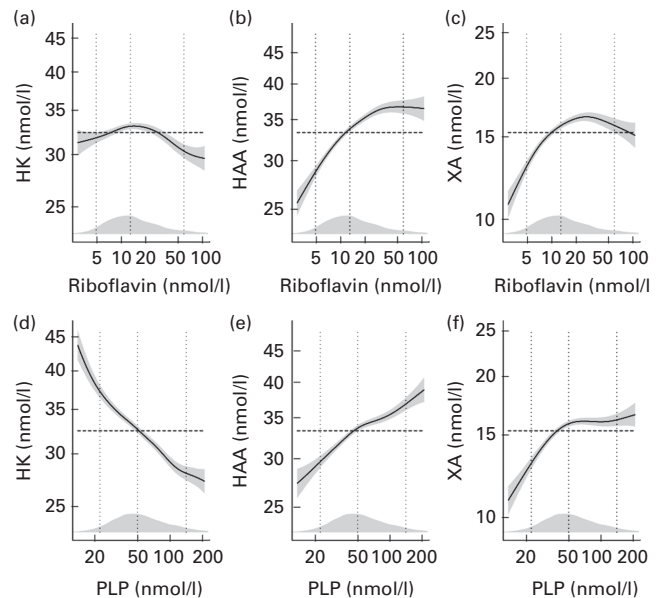
### Strengths and limitations

This is the first epidemiological study to investigate the associations of vitamin B<sub>2</sub> and B<sub>6</sub> status with kynurenines, KTR and neopterin in an apparently healthy population. Although the cross-sectional design of the present study is not appropriate to address causality, the large sample size and comprehensive data including a large panel of kynurenines, vitamin B<sub>2</sub> and B<sub>6</sub> species, and dietary intake provided a unique opportunity to study associations. Data on FAD, the co-enzyme for kynurenine mono-oxygenase, were not available, but are not expected to add important information, as the available forms of riboflavin and FMN are better indicators of vitamin B<sub>2</sub> status<sup>(19)</sup>. The investigated plasma metabolites were stored at  $-80^{\circ}\text{C}$  in EDTA plasma, which is the recommended

temperature for ensuring stability during long-term storage<sup>(28)</sup>. Plasma concentrations of kynurenines<sup>(14)</sup>, and vitamin B<sub>2</sub><sup>(29)</sup> and B<sub>6</sub> species<sup>(14,29)</sup> are similar to those previously reported in other large cohort studies, which validates data integrity. One limitation of the study is the lack of measures of intracellular concentrations of metabolites.

### Vitamin B<sub>2</sub> as a determinant of kynurenines

The relationship of vitamin B<sub>2</sub> with plasma kynurenines was investigated for the first time in human subjects.



**Fig. 2.** Dose-response curves with 95% CI (shaded areas) for the associations of plasma riboflavin and pyridoxal 5'-phosphate (PLP) with plasma (a, d) 3-hydroxykynurenine (HK), (b, e) 3-hydroxyanthranilic acid (HAA) and (c, f) xanthurenic acid (XA). Associations were modelled using generalised additive models. Analyses were adjusted for age, sex and renal function. Both outcome and exposure variables were log-transformed before analysis, and back-transformed values are presented. The top 1% of observations of riboflavin and PLP was excluded from the analysis ( $n = 6851$  for HK and  $n = 6861$  for all other metabolites). Density plots of the distributions of riboflavin and PLP are included in each graph. The 5th, 50th and 95th percentiles of each vitamin are indicated by the vertical dotted lines, and the median of the outcome variable is depicted by the horizontal dashed line.

**Table 3.** Results from segmented regression (SEG)/linear regression with plasma concentrations of riboflavin and pyridoxal 5'-phosphate (PLP) as determinants of tryptophan and kynurenines (Kyn) for participants in the Hordaland Health Study\* ( $\beta$ -Coefficients and 95% confidence intervals)

	$\beta$	95% CI	Breakpoint (nmol/l)	95% CI	Slope 2	95% CI	Model
<b>Riboflavin</b>							
Trp	0.09	0.07, 0.11	12.6	10.6, 14.9	-0.01	-0.02, 0.00	SEG
Kyn	0.03	0.02, 0.04	22.4	13.5, 37.7	-0.01	-0.04, 0.01	SEG
AA	0.06	0.04, 0.07	25.8	13.9, 48.4	0.01	-0.03, 0.04	SEG
KA	0.06	0.05, 0.06					MLR
HK	0.05	0.02, 0.08	14.0	10.9, 17.8	-0.06	-0.08, -0.04	SEG
HAA	0.20	0.16, 0.23	12.2	10.1, 14.7	0.05	0.02, 0.07	SEG
XA	0.24	0.19, 0.28	13.5	11.8, 15.6	-0.02	-0.05, 0.01	SEG
<b>PLP</b>							
Trp	0.15	0.12, 0.18	44.3	38.5, 50.1	0.03	0.02, 0.05	SEG
Kyn	-0.01	-0.02, 0.00					MLR
AA	0.04	0.03, 0.05					MLR
KA	0.18	0.13, 0.23	39.3	32.1, 47.9	0.04	0.02, 0.07	SEG
HK	-0.59	-0.85, -0.33	18.9	16.1, 22.2	-0.15	-0.16, -0.13	SEG
HAA	0.17	0.12, 0.21	47.0	33.1, 66.0	0.08	0.05, 0.11	SEG
XA	0.32	0.25, 0.38	42.5	37.7, 48.4	0.01	-0.02, 0.05	SEG

AA, anthranilic acid; KA, kynurenic acid; MLR, multiple linear regression; HK, 3-hydroxykynurenine; HAA, 3-hydroxyanthranilic acid; XA, xanthurenic acid.

\* All models were adjusted for age, sex and renal function. Both outcome and exposure variables were log-transformed before analysis. The breakpoints were back-transformed and corresponded to vitamin species concentrations (nmol/l). The top 1% of observations of riboflavin and PLP was excluded from analysis ( $n$  6851 for HK and  $n$  6861 for all other metabolites). All metabolites were measured in plasma.

Plasma concentrations of xanthurenic acid and 3-hydroxyanthranilic acid were positively related to both riboflavin and PLP. Thus, the concentrations of these two metabolites seem to be affected by the availability of cofactors (FAD or PLP) for the enzymes kynurenine mono-oxygenase, kynureninase and kynurenine aminotransferases (Fig. 1). Notably, plasma concentration of 3-hydroxykynurenine, the immediate downstream metabolite of kynurenine and the common precursor for both xanthurenic acid and 3-hydroxyanthranilic acid, was not associated with riboflavin, but increased markedly as a function of decreasing PLP concentration. This finding suggests that plasma 3-hydroxykynurenine is primarily determined by its rate of removal rather than formation.

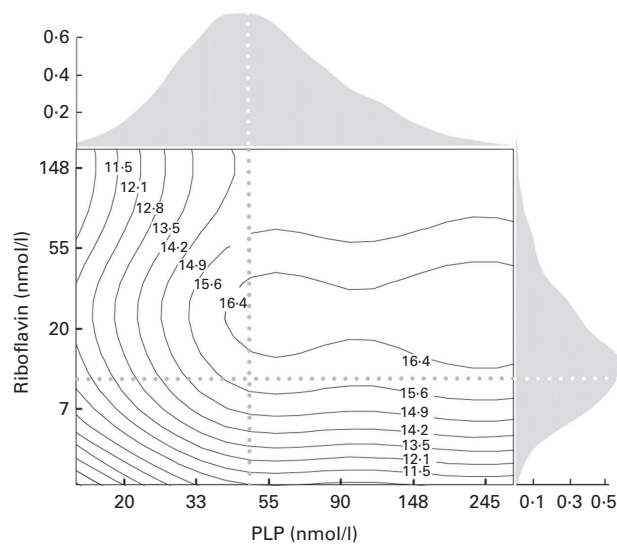
#### Vitamin B<sub>6</sub> as a determinant of kynurenines

The inverse relationship of PLP with 3-hydroxykynurenine is in line with a previous large-scale study on patients with stable angina pectoris<sup>(14)</sup>. However, in the present study, this association was not confined to participants with elevated inflammatory markers<sup>(14)</sup>. Contrasting findings may be accounted for by differences between study populations, as all stages of atherosclerosis including stable angina pectoris are associated with low-grade inflammation<sup>(30)</sup>. In addition, PLP was positively associated with all other kynurenines and inversely associated with KTR and neopterin, which is in agreement with previous reports<sup>(14,15,31)</sup>. The inverse association between PLP and inflammation has been attributed to the mobilisation of PLP to the site of inflammation for use in different immune-related pathways, including the kynurenine pathway<sup>(32)</sup>.

#### Interaction effects of vitamins B<sub>2</sub> and B<sub>6</sub> as determinants of kynurenines

Lastly, xanthurenic acid, but not 3-hydroxyanthranilic acid, was interactively determined by PLP and riboflavin.

This suggests different regulations of the enzymes kynurenine aminotransferase and kynureninase, the enzymes catabolising 3-hydroxykynurenine to xanthurenic and 3-hydroxyanthranilic acid, respectively, possibly related to their different localisation and kinetic properties. Kynurenine aminotransferase



**Fig. 3.** Contour plot for the variations in plasma xanthurenic acid according to the plasma concentrations of riboflavin and pyridoxal 5'-phosphate (PLP). The curves show the contour lines for given concentrations of xanthurenic acid (nmol/l). Contour lines that are close together indicate a steeper variation in xanthurenic acid. Probability density plots of riboflavin and PLP are depicted on the side of the main panel with dotted lines corresponding to the median concentrations. Positive associations of both riboflavin and PLP with xanthurenic acid are essentially confined to subjects with concentrations of the other vitamin below the median value. Xanthurenic acid, riboflavin and PLP were log-transformed before analysis, and back-transformed values are presented. The model was adjusted for age, sex and renal function ( $n$  6931). Data for riboflavin and PLP concentrations between the 1st and 99th percentiles are shown.

is a partly mitochondrial enzyme with a  $K_m$  of  $7 \times 10^{-6}$  for PLP, while kynureninase is exclusively located in the cytosol with a  $K_m$  of  $9 \times 10^{-7}$  (33).

### Conclusions

The present study demonstrates that plasma concentrations of vitamins B<sub>2</sub> and B<sub>6</sub> are determinants of several kynurenines, in a large sample of apparently healthy individuals. The results confirm previous observations that PLP is inversely related to plasma 3-hydroxykynurenine, but positively associated with other kynurenines<sup>(14,15)</sup>. In addition, it is shown that the association between plasma 3-hydroxykynurenine and PLP is independent of riboflavin, an observation that strengthens the case of 3-hydroxykynurenine as a metabolic marker of functional B<sub>6</sub> status. For the first time, it is shown that the metabolites downstream of 3-hydroxykynurenine; xanthurenic acid and 3-hydroxyanthranilic acid, were positively related to riboflavin, with riboflavin and PLP acting as interactive determinants of xanthurenic acid. These results demonstrate the significance and complexities of vitamin B<sub>2</sub> and B<sub>6</sub> status in the kynurenine pathway. Our findings motivate the incorporation of measurements of vitamin B<sub>2</sub> and B<sub>6</sub> status in the increasing number of epidemiological studies<sup>(1,6,34)</sup>, assessing the role of the kynurenine pathway in health and disease development.

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The authors' contributions are as follows: G. S. T., S. E. V., P. M. U. and O. N. designed the study; Ø. M. was responsible for the measurements of the biomarkers; G. S. T. provided essential data; D. T., A. U. and S. J. P. M. E. analysed the data; Ø. M. and S. H. contributed to the interpretation of the data; D. T. and S. J. P. M. E. wrote the first draft of the paper; A. U., Ø. M., P. M. U., G. S. T., S. E. V., O. N. and S. H. provided critical revision of the draft; D. T. and S. J. P. M. E. had primary responsibility for the final content. All authors read and approved the final manuscript.

None of the authors has any conflict of interest to declare.

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