Tryptophan catabolites as metabolic markers of vitamin B6 status evaluated in cohorts of healthy adults and cardiovascular patients

Arve Ulvik, Øivind Midttun, Adrian McCann, Klaus Meyer, Grethe Tell, Ottar Nygård, Per M Ueland

Bevital, Bergen, Norway (AU, ØM, AM, KM)

Department of Global Public Health and Primary Care, University of Bergen, Norway (GT)

Department of Heart Disease, Haukeland University Hospital, Bergen, Norway (ON)

Department of Clinical Science, University of Bergen, Bergen, Norway (PMU)

Corresponding author:

Arve Ulvik,

Bevital, Laboratory building 9th floor

Jonas Lies veg 87, 5021 Bergen, Norway

Phone: +47 55974657

E-mail: arve.ulvik@bevital.no

Sources of support: None

Running head: Kynurenines as metabolic markers of vitamin B6 status

Names for PubMed indexing: Ulvik, Midttun, McCann, Meyer, Tell, Nygård, Ueland

Abbreviations: AA, anthranilic acid; CRP, C-reactive protein; GAM, generalized additive models; HAA, 3-OH anthranilic acid; HK, 3-OH kynurenine; IDO, indoleamine 2,3-

dioxygenase; INF-γ, interferon-γ; KA, kynurenic acid; KAT, kynurenine transaminase; KMO, kynurenine monooxygenase; Kyn, kynurenine; KTR, kynurenine/tryptophan ratio; TDO, tryptophan 2,3-dioxygenase; XA, xanthurenic acid.

ABSTRACT

Background: Vitamin B6 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 B6 status impacts the kynurenine pathway of tryptophan catabolism.

Objective: To comprehensively evaluate the use of kynurenines as potential markers of functional vitamin B6 status across two large cohorts.

Design: We measured circulating concentrations of the first 6 metabolites in the tryptophan 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 kynurenines was estimated using linear and non-linear regression-based methods.

Results: 3´-Hydroxykynurenine (HK), a substrate, and all four 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´-hydroxy anthranilic acid + anthranilic acid) termed HK-ratio (HKr) was related to plasma PLP with standardized regression coefficients (95% CI) 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 - 2.7 fold more sensitive, but also 1.7 - 2.9 fold more specific to changes in PLP compared to a previously proposed marker HK:xanthurenic acid (HK:XA). Notably, the association was strongest at PLP concentrations < ~20 nmol/L, a recognized threshold for vitamin B6 deficiency. Finally, PLP and HKr demonstrated highly sex-specific and corroborating associations with age.

Conclusions: The results demonstrate that by combining five metabolites in the kynurenine pathway into a simple index, HKr, a sensitive and specific indicator of intracellular vitamin B6 status is obtained. The data also underscores the merit of evaluating alterations in kynurenine metabolism when investigating vitamin B6 and

health.

31 Keywords: Vitamin B6, nutritional status, biomarker, inflammation, metabolic,

32 functional

33

INTRODUCTION

The involvement of vitamin B6 in human metabolism includes the synthesis and interconversion of amino acids, neurotransmitters, nucleic acids, heme, and lipids. Vitamin B6 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 B6, is underscored by its use as a coenzyme in all the major enzyme classes except for ligases (1). Both vitamin B6 intake and plasma indicators of vitamin B6 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).

One of the earliest described indicators of low vitamin B6 status was the increased excretion of the tryptophan catabolite xanthurenic acid (XA) in urine after a tryptophan load (6). Subsequently, a number of metabolites along the kynurenine pathway of tryptophan catabolism were found to be increased in the urine of vitamin B-6 deficient humans, including the ratio of 3´ hydroxykynurenine:3`hydroxyanthranilic acid (HK:HAA) (7,8). An overview of tryptophan metabolism and its two PLP-dependent steps is shown in **Figure 1**. Methods and protocols for quantification of these and other functional markers of vitamin B6 status are often cumbersome, however, and have largely been abandoned after sensitive and precise measurements of plasma PLP became available (9,10). Although plasma PLP is accepted as an indicator of nutritional vitamin B6 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 B6 vitamers (PLP, pyridoxal (PL), and 4´-pyridoxic acid (PA)) in serum/plasma to also include tryptophan and the first 6 metabolites of the tryptophan degradation pathway. In a cohort of suspected coronary artery disease (CAD) patients we noted that HK was markedly increased at plasma PLP concentrations below 20 nmol/L, a cut-off suggested to indicate vitamin B6 deficiency (12). In a follow-up study we evaluated substrate:product ratios of

the two 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 to HK alone (13). Subsequently, this, and other kynurenine-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 (J Nut, in press).

Previously, we evaluated kynurenines in a cohort of confirmed and suspected coronary artery disease (CAD) patients (13). The objective of the present study was to perform an in-depth exploration of the concept of kynurenines as metabolic markers of vitamin B6 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).

SUBJECTS AND METHODS

Study populations

The Hordaland Health Study (HUSK) is a community-based longitudinal observational study whose baseline measurements were conducted during 1997-1999 (http://husk.b.uib.no). Details of the study design and methodology have been described elsewhere (17,18). 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 kynurenines, 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 (19). About 2/3 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 four treatment groups consisted of 1) 0.8 mg folic acid, 0.4 mg cyanocobalamin and 40 mg pyridoxin, 2) 0.8 mg folic acid,

0.4 mg cyanocobalamin, 3) 40 mg pyridoxin,and 4) placebo in a 2x2 factorial design. WENBIT is described in detail elsewhere (20). After exclusion of 45 participants with missing data on PLP and kynurenines, 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 kynurenines across 28 days. Complete data for 2508 participants were available for this analysis. Participant flow charts for HUSK and WECAC are available as **Supplemental Figure 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 non-smokers, but with plasma cotinine ≥ 85 nmol/L (21) were re-classified as smokers. Height and weight were measured using standardized protocols, and body mass index (BMI) was calculated by dividing weight by height squared (kg/m^2).

Laboratory analyses

Non-fasting blood samples were collected into tubes containing EDTA, kept on ice before centrifugation (within 3 hr), and stored at -80°C before analysis. Plasma concentrations of PLP, tryptophan, kynurenines, neopterin, cotinine, and creatinine were quantified by liquid chromatography/tandem mass spectrometry at Bevital, Bergen, Norway (www.bevital.no) (22,23). C-reactive protein (CRP) was measured in serum using an ultrasensitive immunoassay, Behring nephelometer II system N Latex CRP mono (Behring Diagnostics, Marburg, Germany) (WECAC) or in plasma with an immuno-MALDI based assay (HUSK) (24). Further details concerning handling and storage of blood samples before analysis (WECAC) have been described previously (19,25,26).

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 Mann-Whitney U test for continuous, and Fisher´s exact test for categorical data. The correlation between kynurenines were estimated by Pearson´s r adjusted for age and sex. Linear- and non-linear associations between vitamin B6 markers and PLP were evaluated by multivariable linear regression, generalized additive models (GAM), and segmented regression. Subcohorts were based on dichotomous variables, or, if continuous, above vs. below the median. Data was divided into low and high inflammation according to the median of the product of CRP, neopterin, and the kynurenine:tryptophan ratio (KTR) . Predictors of vitamin B6 markers were evaluated using "relative importance regression". This method combines multiple linear regression with the algorithm "lmg" as described (27). 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 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 indices 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 by similar terms used in receiver operating curve (ROC) analysis. We used R for Macintosh version 3.5.2 for all statistical calculations, with R-packages "mgcv" for GAM, "segmented" for segmented regression, and "relaimpo" for the multiple linear regression-based assessment of relative importance of predictors.

RESULTS

Characteristics of the study populations

149 The HUSK cohort consisted of two distinct age groups: 46-47 years (52.8%) and 70-72
150 years, with ~56% women in each age group. In WECAC, the median (IQR) age was 61
151 (14) years for men, and 64 (15) years for women, with 28.1% women. The concentrations
152 of tryptophan and the kynurenines were similar in HUSK and WECAC (**Table 1**). In both
153 cohorts, the concentrations of Trp, Kyn, KA, XA and HAA were higher in men than
154 women. PLP concentrations were similar across genders, but both HK:XA and the ratio
155 HKr = HK:(KA+XA+HAA+AA) were higher in women (Table 1). In WECAC, 2125
156 patients (52%) had previously established cardiovascular disease, and 3082 (75%) had 1
157 or more stenotic vessels based on coronary angiography.
158
159 *Initial exploration of the relation between PLP and kynurenines*
160 We modeled PLP by linear regression and stepwise selection using the kynurenines
161 downstream of Kyn as candidate predictors while keeping age and sex as fixed
162 covariates. In both HUSK and WECAC, HK was selected as the first (and only negative)
163 predictor of PLP followed by all four of XA, KA, HAA, and AA (postitive predictors). In
164 unadjusted analyses, we confirmed that the proportions of KA, XA, HAA, and AA all
165 increased while HK decreased across quartiles of PLP. The correlations (Pearson´s r
166 adjusted for age and sex) of KA, XA, HAA, and AA with their sum were 0.85, 0.76, 0.49,
167 and 0.79, respectively in HUSK, and 0.88, 0.80, 0.52, and 0.71 in WECAC. Inspired by
168 these results, the ratio HKr = HK:(KA+XA+AA+HAA) was constructed as a candidate
169 marker of vitamin B6 status. We also included ratios aimed at specifically characterizing
170 kynurenine aminotranferase (KAT) denoted HK_{KAT} = HK/(KA+XA), and kynureninase
171 (KYNU): HK_{KYNU} = HK/(AA+HAA). In the following we will refer to kynurenines,
172 either singly or in combination as (potential) functional vitamin B6 markers.
173
174 *Linear associations between PLP and selected vitamin B6 markers*
175 We evaluated the linear association of PLP with vitamin B6 markers by multiple linear
176 regression adjusted for age and sex (**Table 2**). Notably, kynurenine combinations (ratios)
177 were more strongly associated with PLP than individual metabolites, and HKr

178 demonstrated the strongest association with PLP in both cohorts. The associations of PLP
179 with Kyn and the ratios Kyn:KA and Kyn:AA were all weak (standardized betas > -
180 0.14). When evaluated in strata based on sex, age, vitamin supplement use, and
181 inflammation (both cohorts) and according to established CVD at baseline and ≥ 1
182 stenotic vessel (WECAC), HKr was consistently the best marker in terms of strength of
183 association with PLP.
184
185 *Determinants of vitamin B6 markers*
186 We evaluated the association of selected vitamin B6 markers with age, BMI, current
187 smoking, kidney function (creatinine), inflammation, as represented by the three variables
188 CRP, neopterin, and KTR, and PLP using relative importance regression stratified by sex.
189 Results for the markers HK, HK:XA and HKr in WECAC are shown in **Figure 2** and
190 relative performances of the markers by cohort and sex are summarized in **Table 3**. As
191 demonstrated in Figure 2 and Table 3, both the sensitivity and specificity for PLP
192 increased in the direction of more complex ratios, and, again, except for specificity in
193 WECAC females, HKr was the best scoring marker in all strata (Table 3). In addition to
194 the markers included in Table 3 we also evaluated a construct where the four downstream
195 kynurenines were standardized before summation, and another construct where we used
196 the product instead of the sum of the four downstream kynurenines. Compared to HKr,
197 the performance of these alternative markers were considerable poorer by the criteria
198 used in Table 3.
199
200 *The association of vitamin B6 markers with PLP by generalized additive models*
201 *regression (GAM)*
202 **Figure 3** shows the association of HK, HK:XA and HKr with PLP in HUSK by GAM.
203 Corresponding, and very similar, results were found in WECAC (**Supplemental Figure**
204 **3**). Common to all markers was a markedly stronger association at low compared to
205 normal and high PLP concentrations. Using segmented regression, we identified a
206 breakpoint at 19.4 (18.1, 20.7) nmol/L PLP for the HKr-PLP association in HUSK and a

similar breakpoint 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 approximately 20 - 40 nmol/L in both cohorts, but we were unable to obtain reproducible breakpoints for a possible intermediate segment by segmented regression.

Longitudinal associations

For WECAC patients that participated in the WENBIT trial we had data on PLP and vitamin B6 markers at baseline, and the 28 days study visit. Moreover, one arm of the 2x2 factorial RCT design included a daily oral dose of 40 mg pyridoxine. Thus, we were able to assess the change in vitamin B6 markers according to both natural variation in PLP (across 28 days) and, according to pyridoxine treatment. **Figure 4** shows GAM-plots of the change in HKr vs. change in PLP in the non-treated and pyridoxine-treated groups. Standardized linear regression coefficients for the associations in the non-treated, 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 B6 treated groups were 39, 31, and 34%, respectively. The above 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 B6 status with age among WECAC men as indicated by both PLP and HKr. For women, B6-status appeared to improve until age 55 and then declined at an increasing rate at age > 55 years. 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 kynurenines as potential functional markers of vitamin B6 status in one community-based and one clinical cohort. Among the panel of candidate markers, the best performance characteristics were found for the ratio HK:(KA+XA+HAA+AA), abbreviated HKr. Compared with kynurenine-based markers proposed earlier (13) and additional markers included in this study, HKr demonstrated stronger associations with PLP, both in cross-sectional and longitudinal analyses, and also considerably increased specificity for PLP. The findings were consistent across cohorts and subgroups and featured a two-segmented dose-response curve with a cut-off close to 20 nmol/L, a threshold suggested to indicate B6 deficiency (28,29).

Possible mechanisms

Previously, we reported the characteristics of the two 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 (30). Briefly, by taking ratios, the influence of confounders common to the nominator and denominator would tend to be attenuated, whereas information related to the 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 four kynurenines downstream of the two 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 kynurenines, 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 cohort and gender. 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 mono-oxygenase (KMO) and thus does not accumulate as PLP becomes limiting. Riboflavin status has been shown to affect the activity of KMO (31), but did not materially affect the relation between PLP and B6 markers in the present study. The mean concentrations of KA, XA, HAA and AA differed by up to 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 kynurenines. Notably, a construct using the product of downstream kynurenines in the denominator was inferior to HKr. Similarly, replacing the downstream kynurenines 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, kynurenine.

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 B6 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 cutpoints for overt, and marginal vitamin B6 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 B6 status, but, conversely, it can also be viewed as novel and direct metabolic support for a cutpoint of 20 nmol/L for vitamin B6 deficiency. Several investigators have studied a related concept of marginal deficiency defined as PLP

293 concentrations in the interval 20 - 30 nmol/L (32). In the GAM analyses there was some
294 support for a segment in the interval 20 - 40 nmol/L PLP where the association with HKr
295 was intermediate. The HKr decreased further beyond 40 nmol/L PLP, thus, it would be
296 hard to use the present data to argue for a specific threshold for sub-optimal vitamin B6
297 status. Although, the data only offers limited support, it certainly does not conflict with a
298 concept of marginal vitamin B6 deficiency in an interval stretching from 20 to 30 nmol/L
299 PLP or even above.
300
301 *HKr and differences according to age and sex*
302 HKr was markedly higher in women than in men in both HUSK and WECAC (13, and
303 18% higher respectively, age-adjusted), while corresponding values for PLP were 4%
304 higher and 5% lower. Notably, both KAT and KYNU has been found to be inhibited by
305 estrogen (33) which could explain the lower concentrations of KA, XA, and HAA, and
306 therefore higher HKr, in women. Declining estrogen levels (34) could also, potentially,
307 explain the downward trend in HKr until about 55 years. Notably, however, high
308 estrogen, e.g. from oral contraceptives, or around the time of ovulation, is associated with
309 low PLP (29). Thus, the similarity of the PLP- and HKr-age assocation curves suggests
310 that the age-related differences in HKr, is mediated through changes in PLP rather than
311 resulting from direct effects of estrogen on KAT and KYNU. In men, and in women older
312 than 55 years, vitamin B6 status decreased according to both indicators. The rate of
313 decline corresponded well with a previously published value of 4 nmol/decade (35) and
314 with other reports (36). Possible explanations could include increased inflammation
315 and/or age-related differences in nutrition (3,36).
316
317 *Strength and limitations*
318 The main strengths of the study included the use of an established mass-spectrometry
319 based assay that quantifies tryptophan, all the kynurenines, and PLP in a single run. We
320 were able to use data from two large cohorts with notable differences in characteristics to
321 assess reproducibility and consistency. Furthermore, data from WENBIT participants

allowed us to evaluate both longitudinal aspects and responses to intervention with vitamin B6 (pyridoxin). The main limitation was the lack of a third, independent, marker of vitamin B6 status. We could only evaluate the kynurenine-based markers against plasma PLP.

Conclusions

In this paper we describe an in-depth exploration of circulating kynurenines as functional markers of vitamin B6 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 two PLP-dependent enzymes in the kynurenine 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 kynurenines 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 (37,38). Since low vitamin B6 status has been found for many of the same conditions (10, 11), it should be of great value to jointly investigate kynurenines and vitamin B6 status in future studies of clinical outcomes.

ACKNOWLEDGEMENTS

Conflict of interest: None. Author contributions: AU, ON, GT and PMU designed research; ØM, AM and KM conducted research; AU performed statistical analysis; AU wrote the paper. AU had primary responsibility for the final content. All authors read and approved the final manuscript.

REFERENCES

1. Percudani R, and Peracchi A. The B6 database: a tool for the description and classification of vitamin B6-dependent enzymatic activities and of the corresponding protein families. BMC Bioinformatics 2009;10:273.

2. Ulvik A, Pedersen ER, Svingen GF, McCann A, Midttun Ø, Nygård O, and Ueland PM. Vitamin B-6 catabolism and long-term mortality risk in patients with coronary artery disease. Am J Clin Nutr 2016;103:1417-25.

3. Lotto V, Choi SW, and Friso S. Vitamin B6: a challenging link between nutrition and inflammation in CVD. Br J Nutr 2011;106:183-95.

4. Kelly PJ, Kistler JP, Shih VE, Mandell R, Atassi N, Barron M, Lee H, Silveira S, and Furie KL. Inflammation, homocysteine, and vitamin B6 status after ischemic stroke. Stroke 2004;35:12-5.

5. Johansson M, Relton C, Ueland PM, Vollset SE, Midttun Ø, Nygård O, Slimani N, Boffetta P, Jenab M, Clavel-Chapelon F et al. Serum B vitamin levels and risk of lung cancer. JAMA 2010;303:2377-85.

6. Linkswiler H. Biochemical and physiological changes in vitamin B6 deficiency. Am J Clin Nutr 1967;20:547-61.

7. Coon WW, and Nagler E. The tryptophan load as a test for pyridoxine deficiency in hospitalized patients. Ann N Y Acad Sci 1969;166:30-43.

8. O´Brien D, and Jensen C. Pyridoxin dependency in two mentally retarded subjects. Clin Sci. 1963;24:179-86.

9. Lamers Y. Indicators and methods for folate, vitamin B-12, and vitamin B-6 status assessment in humans. Curr Opin Clin Nutr Metab Care 2011;14:445-54.

10. Ueland PM, Ulvik A, Rios-Avila L, Midttun Ø, and Gregory JF. Direct and Functional Biomarkers of Vitamin B6 Status. In Annu Rev Nutr 2015 p33-70.

11. Paul L, Ueland PM, and Selhub J. Mechanistic perspective on the relationship between pyridoxal 5'-phosphate and inflammation. Nutr Rev 2013;71:239-44.

12. Midttun O, Ulvik A, Ringdal Pedersen E, Ebbing M, Bleie O, Schartum-Hansen H, Nilsen RM, Nygard O, and Ueland PM. Low plasma vitamin B-6 status affects

metabolism through the kynurenine pathway in cardiovascular patients with systemic inflammation. J Nutr 2011;141:611-7.

13. Ulvik A, Theofylaktopoulou D, Midttun Ø, Nygård O, Eussen SJ, and Ueland PM. Substrate product ratios of enzymes in the kynurenine pathway measured in plasma as indicators of functional vitamin B-6 status. Am J Clin Nutr 2013;98:934-40.

14. Theofylaktopoulou D, Midttun Ø, Ueland PM, Meyer K, Fanidi A, Zheng W, Shu XO, Xiang YB, Prentice R, Pettinger M et al. Impaired functional vitamin B6 status is associated with increased risk of lung cancer. Int J Cancer 2018;142:2425-2434.

15. Gylling B, Myte R, Schneede J, Hallmans G, Häggström J, Johansson I, Ulvik A, Ueland PM, Van Guelpen B, and Palmqvist R. Vitamin B-6 and colorectal cancer risk: a prospective population-based study using 3 distinct plasma markers of vitamin B-6 status. Am J Clin Nutr 2017;105:897-904.

16. Minović I, van der Veen A, van Faassen M, Riphagen IJ, van den Berg E, van der Ley C, Gomes-Neto AW, Geleijnse JM, Eggersdorfer M, Navis GJ, Kema IP, and Bakker SJ. Functional vitamin B-6 status and long-term mortality in renal transplant recipients. Am J Clin Nutr 2017;106:1366-74.

17. Refsum H, Nurk E, Smith AD, Ueland PM, Gjesdal CG, Bjelland I, Tverdal A, Tell GS, Nygard O, and Vollset SE. The Hordaland Homocysteine Study: a community-based study of homocysteine, its determinants, and associations with disease. J Nutr 2006;136:1731S-40S.

18. Vikse BE, Vollset SE, Tell GS, Refsum H, and Iversen BM. Distribution and determinants of serum creatinine in the general population: the Hordaland Health Study. Scand J Clin Lab Invest 2004;64:709-22.

19. Svingen GF, Ueland PM, Pedersen EK, Schartum-Hansen H, Seifert R, Ebbing M, Løland KH, Tell GS, and Nygård O. Plasma dimethylglycine and risk of incident acute myocardial infarction in patients with stable angina pectoris. Arterioscler Thromb Vasc Biol 2013;33:2041-8.

20. Ebbing M, Bleie O, Ueland PM, Nordrehaug JE, Nilsen DW, Vollset SE, Refsum H, Pedersen EK, and Nygard O. Mortality and cardiovascular events in patients treated with

homocysteine-lowering B vitamins after coronary angiography: a randomized controlled trial. JAMA 2008;300:795-804.

21. Jarvis MJ, Tunstall-Pedoe H, Feyerabend C, Vesey C, and Saloojee Y. Comparison of tests used to distinguish smokers from nonsmokers. Am J Public Health 1987;77:1435-8.

22. Midttun O, Hustad S, and Ueland PM. Quantitative profiling of biomarkers related to B-vitamin status, tryptophan metabolism and inflammation in human plasma by liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 2009;23:1371-9.

23. Midttun O, Kvalheim G, and Ueland PM. High-throughput, low-volume, multianalyte quantification of plasma metabolites related to one-carbon metabolism using HPLC-MS/MS. Anal Bioanal Chem 2013;405:2009-17.

24. Meyer K, and Ueland PM. Targeted quantification of C-reactive protein and cystatin c and its variants by immuno-MALDI-MS. Anal Chem 2014;86:5807-14.

25. Svingen GF, Schartum-Hansen H, Ueland PM, Pedersen ER, Seifert R, Ebbing M, Bønaa KH, Mellgren G, Nilsen DW, Nordrehaug JE, Oyen J, and Nygård O. Elevated plasma dimethylglycine is a risk marker of mortality in patients with coronary heart disease. Eur J Prev Cardiol 2015;22:743-52.

26. Bonaa KH, Njolstad I, Ueland PM, Schirmer H, Tverdal A, Steigen T, Wang H, Nordrehaug JE, Arnesen E, and Rasmussen K. Homocysteine lowering and cardiovascular events after acute myocardial infarction. N Engl J Med 2006;354:1578-88.

27. Groemping U. Relative importance for Linear regression in R: The package realimpo. Journal of Statistical Software. 2006;17.

28. Food and Nutrition Board, Institute of Medicine. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. A report of the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, Other B Vitamins, and Choline and Subcommittee on Upper Reference Levels of Nutrients. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. A report of the Standing Committee on the Scientific Evaluation of Dietary

Reference Intakes and its Panel on Folate, Other B Vitamins, and Choline and Subcommittee on Upper Reference Levels of Nutrients. Washington, DC: National Academy Press, 1998.

29. Morris MS, Picciano MF, Jacques PF, and Selhub J. Plasma pyridoxal 5'-phosphate in the US population: the National Health and Nutrition Examination Survey, 2003-2004. Am J Clin Nutr 2008;87:1446-54.

30. Fell DA. Enzymes, metabolites and fluxes. J Exp Bot 2005;56:267-72.

31. Theofylaktopoulou D, Ulvik A, Midttun Ø, Ueland PM, Vollset SE, Nygård O, Hustad S, Tell GS, and Eussen SJ. Vitamins B2 and B6 as determinants of kynurenines and related markers of interferon-γ-mediated immune activation in the community-based Hordaland Health Study. Br J Nutr 2014;112:1065-72.

32. Gregory JF, Park Y, Lamers Y, Bandyopadhyay N, Chi Y, Lee K, Kim S, da Silva V, Hove N, Ranka S et al. Metabolomic analysis reveals extended metabolic consequences of marginal vitamin B-6 deficiency in healthy human subjects. PLoS ONE 2013;8:e63544

33. Jayawickrama GS, Nematollahi A, Sun G, Gorrell MD, and Church WB. Inhibition of human kynurenine aminotransferase isozymes by estrogen and its derivatives. Sci Rep 2017;7:1-11

34. Lephart ED. A review of the role of estrogen in dermal aging and facial attractiveness in women. J Cosmet Dermatol 2018;17:282-88

35. Rose CS, Gyorgy P, Butler M, Andres R, Norris AH, Shock J, Tobin J, Brin M, and Spiegel H. Age differences in vitamin B6 status of 617 men. Am J Clin Nutr 1976;29:847-53

36. Bates CJ, Pentieva, KD, Prentice A, Mansoor MA, and Finch S. Plasma pyridoxal phosphate and pyridoxic acid and their relationship to plasma homocysteine in a representative sample of British men and women aged 65 years and over. Br J Nutr 1999;81:191-201

37. Chen Y, Guillemin GJ. Kynurenine pathway metabolites in humans: disease and healthy states. Int J Tryptophan Res 2009;2:1-19

38. Savitz, J. The kynurenine pathway: a finger in every pie 2019. Mol Psychiatry 2019;24:1-17

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 46-47y, n (%)	1623 (44.4)	2033 (55.6)				
Age 70-72y, n (%)	1439 (44.0)	1829 (56.0)				
Age (y)				61 (15)	64 (15)	< 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 (4.0)	24.9 (5.5)	< 0.001	26.4 (4.3)	26.1 (6.1)	0.009
Creatinine (mmol/L)	88.5 (15.5)	73.7 (12.7)	< 0.001	77.4 (18.1)	64.8 (16.3)	< 0.001
PLP (nmol/L)	50.5 (35.8)	49.8 (44.4)	0.80	42.0 (29.6)	39.7 (32.2)	0.10
Trp (mmol/L)	70.5 (18.0)	64.3 (17.5)	< 0.001	71.4 (18.7)	66.5 (19.1)	< 0.001
Kyn (mmol/L)	1.59 (0.56)	1.45 (0.56)	< 0.001	1.70 (0.61)	1.63 (0.66)	0.005
HK (nmol/L)	31.9 (14.4)	32.5 (14.8)	0.05	30.1 (15.2)	32.8 (17.6)	< 0.001
KA (nmol/L)	48.9 (23.2)	42.5 (21.3)	< 0.001	50.1 (26.1)	43.0 (21.7)	< 0.001
XA (nmol/L)	16.9 (10.5)	14.7 (9.4)	< 0.001	15.1 (9.9)	12.7 (9.4)	< 0.001
AA (nmol/L)	14.4 (6.7)	14.0 (6.3)	0.001	14.3 (6.9)	14.5 (7.0)	0.64
HAA (nmol/L)	35.2 (17.3)	31.9 (15.7)	< 0.001	35.9 (19.9)	30.7 (16.0)	< 0.001
HK:XA (no units)	1.88 (1.04)	2.19 (1.45)	< 0.001	1.99 (1.08)	2.48 (1.78)	< 0.001
HKr x100 (no units)	26.9 (9.6)	30.1 (12.1)	< 0.001	25.5 (10.1)	31.0 (14.4)	< 0.001
CRP (mg/L)	1.62 (2.8)	1.54 (3.0)	0.03	1.77 (2.70)	1.80 (3.1)	0.07

KTR (nmol/μmol)	22.6 (9.2)	22.5 (9.7)	0.52	23.5 (8.9)	24.8 (10.2)	< 0.001
Neopterin (nmol/L)	7.5 (2.9)	7.8 (3.0)	< 0.001	7.9 (3.4)	8.9 (4.3)	< 0.001

[1]Numbers are medians (IQR) if not otherwise indicated.
[2] Mann Whitney U test or Fisher´s exact test for difference between men and women
AA, anthranilic acid; CRP, C-reactive protein; HAA, 3-OH anthranilic acid; HK, 3-OH kynurenine; HKr, HK:(KA+XA+AA+HAA); KA, kynurenic acid; Kyn, kynurenine; KTR, kynurenine:tryptophan (ratio); PLP, pyridoxal 5´-phosphate; Trp, tryptophan; XA, xanthurenic acid

Table 2. Linear associations between vitamin B6 markers and PLP[1]

	HUSK	WECAC
Single kynurenines		
HK	-26 (-29, -24)	-25 (-28, -22)
KA	13 (10, 15)	13 (10, 16)
XA	13 (10, 15)	17 (14, 20)
AA	7 (4, 9)	10 (7, 13)
HAA	17 (15, 20)	12 (9, 15)
Ratios flanking KAT		
HK:KA	-38 (-41, -36)	-37 (-40, -35)
HK:XA	-33 (-35, -30)	-41 (-44, -38)
HK_{KAT} = HK:(KA+XA)	-41 (-43, -39)	-41 (-44, -38)
Ratios flanking KYNU		
HK:AA	-28 (-30, -26)	-29 (-32, -27)
HK:HAA	-43 (-45, -41)	-38 (-41, -35)
HK_{KYNU} = HK:(AA+HAA)	-43 (-46, -41)	-40 (-43, -38)
Ratio flanking both enzymes		
HKr = HK:(KA+XA+AA+HAA)	-47 (-49, -45)	-46 (-49, -43)

[1]Numbers are standardized regression coefficients x 100 (95% CI) adjusted for age and sex. All associations were significant at $P < 0.0001$. AA, anthranilic acid; HAA, 3-OH anthranilic acid; HK, 3-OH kynurenine; KA, kynurenic acid; KAT, kynurenine transaminase; KYNU; kynureninase; XA, xanthurenic acid.

Table 3. Performance characteristics of selected markers of vitamin B6 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 illustrated in Figure 2.)
AA, anthranilic acid; HAA, 3-OH anthranilic acid; HK, 3-OH kynurenine; KA, kynurenic acid; XA, xanthurenic acid.

LEGENDS TO FIGURES

Figure 1. Tryptophan metabolism through the kynurenine pathway

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

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 with light grey, and black color, respectively. CRP, C-reactive protein; KTR, kynurenine:tryptophan ratio; PLP, pyridoxal 5´-phosphate.

Figure 3. Association of HK, HK:XA and HKr with PLP in HUSK by GAM

Grey 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 percentile. The horizontal dotted line marks the adjusted mean concentration of the vitamin B6 marker. For the HKr-association, two segments, calculated by segmented regression, is overlaid (red color) on the GAM-curve, and a significant breakpoint at 19.4 (18.1, 20.7) nmol/L is indicated by the vertical dotted line. GAM, generalized additive models.

Figure 4. Change in HKr versus change in PLP by GAM

Change is defined as the concentration of the vitamin B6 marker 28 days into the study (WENBIT) divided by the concentration at baseline. A: non-treated groups (n=1130). B: groups treated with a daily oral dose of 40 mg pyridoxine (n=1138). Grey shaded areas denote the 95% CI. The distribution of ΔPLP is shown at the bottom of each panel with white lines indicating the 5th, 20th, 50th, 80th, and 95th percentile. Horizontal and vertical dotted lines indicate where no change from baseline to day 28 is found (ratio = 1).

Figure 5. The association of HKr with age in WECAC by GAM

Grey 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 percentile. The horizontal dotted line in each panel indicate the adjusted mean concentration of the vitamin B6 marker.

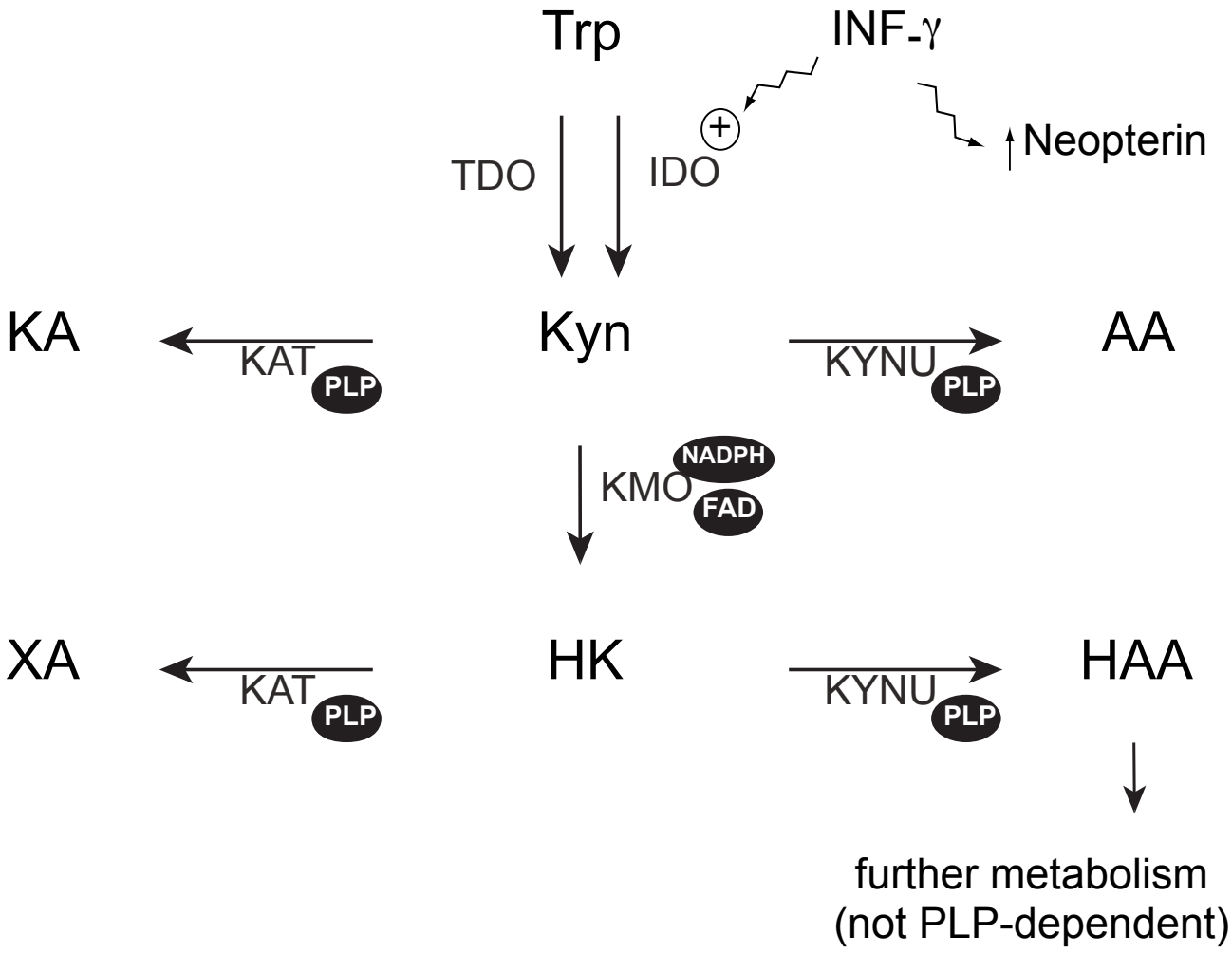
Trp
INF-γ
+
Neopterin
TDO
IDO
KA
KAT
PLP
Kyn
KYNU
PLP
AA
NADPH
KMO
FAD
XA
KAT
PLP
HK
KYNU
PLP
HAA
further metabolism
(not PLP-dependent)

Figure 1

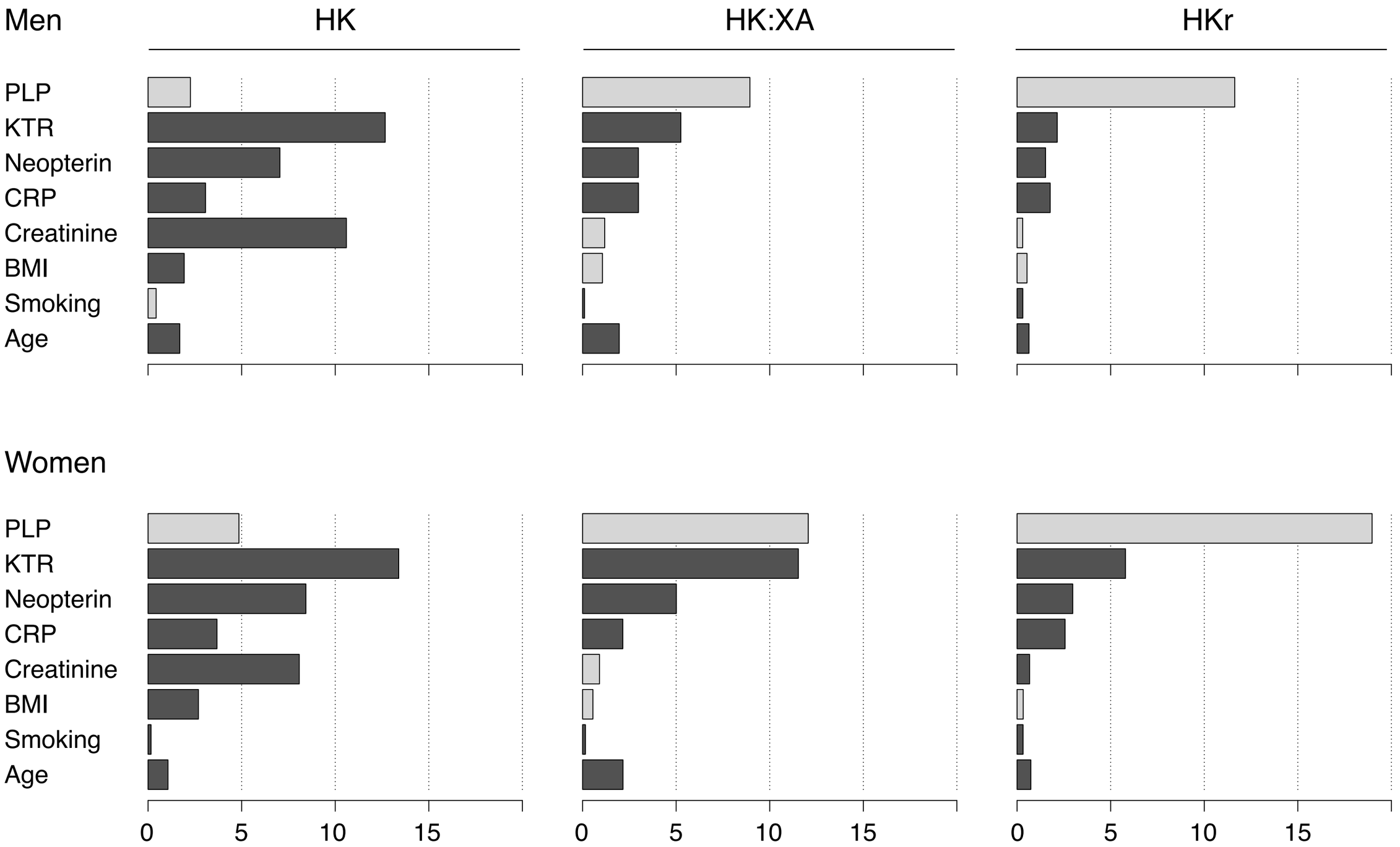
Men
HK
HK:XA
HKr
PLP
KTR
Neopterin
CRP
Creatinine
BMI
Smoking
Age
Women
PLP
KTR
Neopterin
CRP
Creatinine
BMI
Smoking
Age
0
5
10
15
0
5
10
15
0
5
10
15
Variance explained (%)

Figure 2

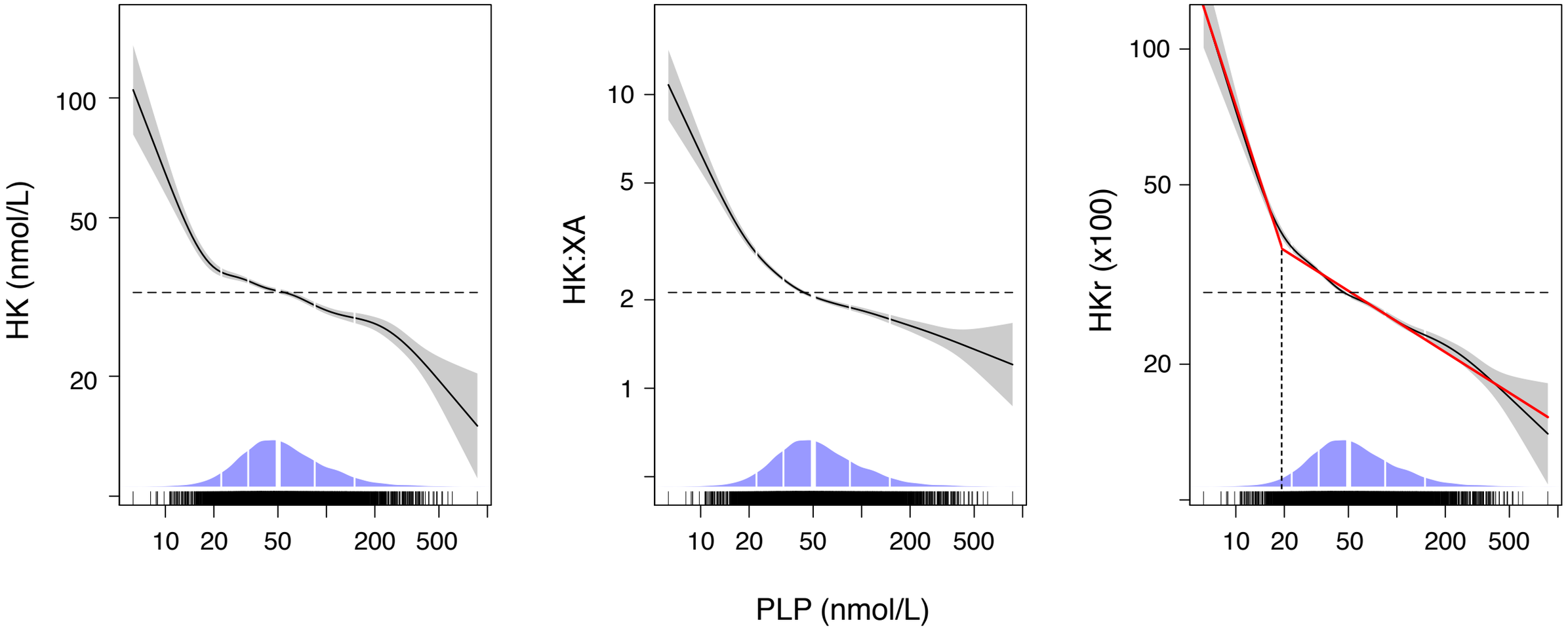
HK (nmol/L)
100
50
20
10
20
50
200
500
HK:XA
10
5
2
1
10
20
50
200
500
PLP (nmol/L)
HKr (x100)
100
50
20
10
20
50
200
500

Figure 3

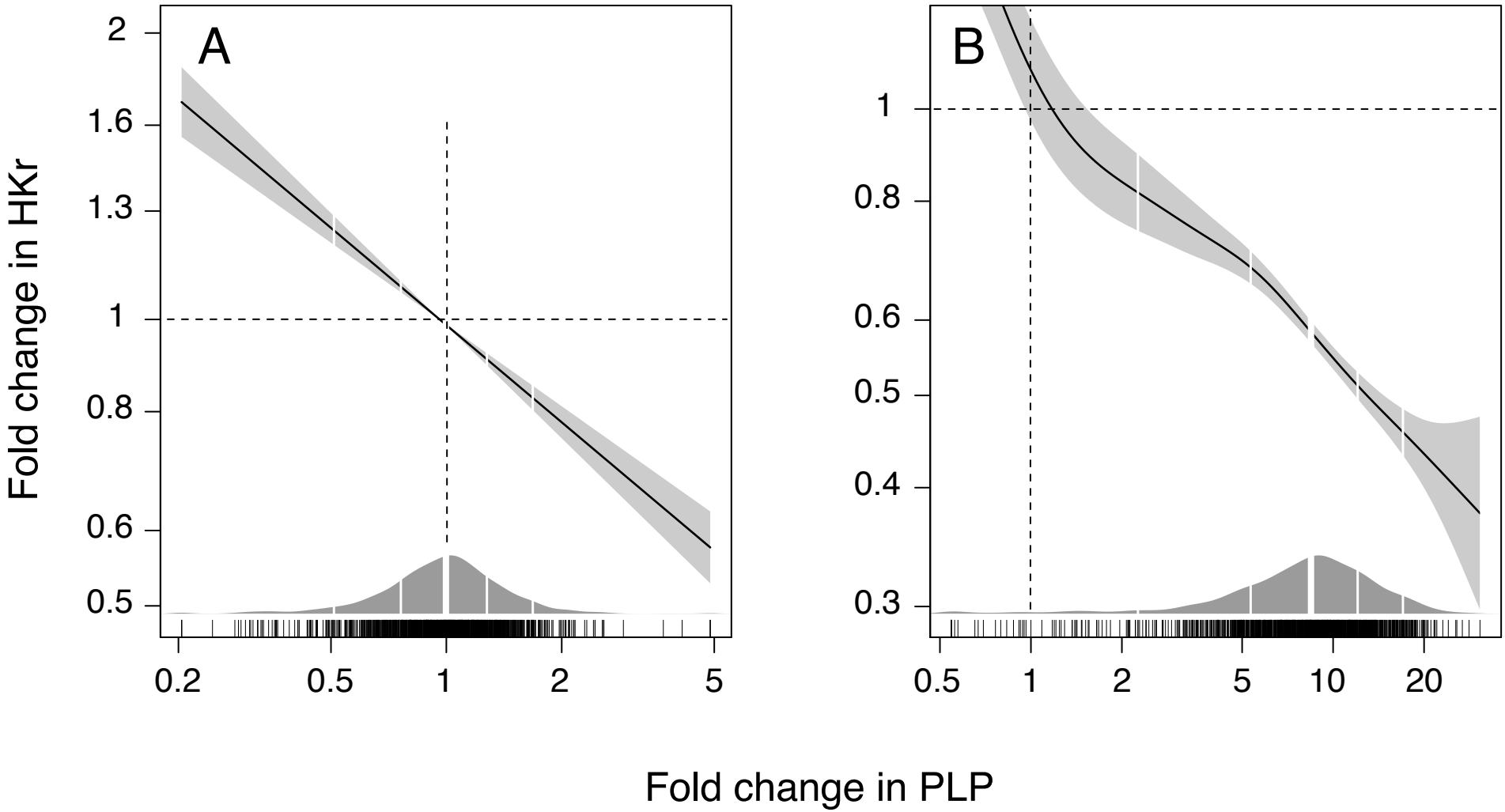
A
B
Fold change in HKr
Fold change in PLP
2
1.6
1.3
1
0.8
0.6
0.5
0.2
0.5
1
2
5
0.3
0.4
10
20

Figure 4

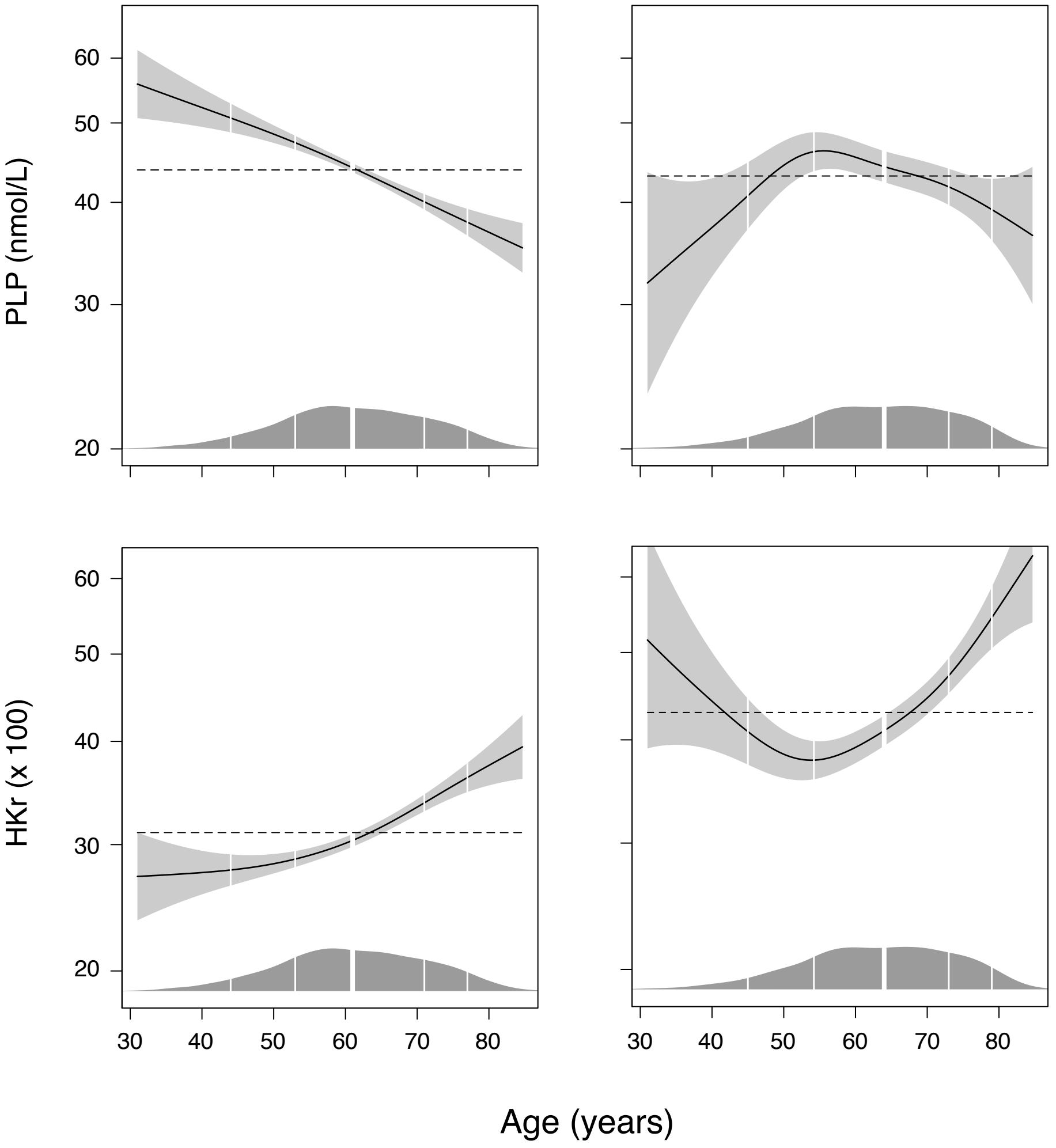
Men
Women
PLP (nmol/L)
60
50
40
30
20
HKr (x 100)
60
50
40
30
20
30
40
50
60
70
80
Age (years)

Figure 5