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Author/s:

Theofylaktopoulou, D;Midttun, Ø;Ueland, PM;Meyer, K;Fanidi, A;Zheng, W;Shu, XO;Xiang, YB;Prentice, R;Pettinger, M;Thomson, CA;Giles, GG;Hodge, A;Cai, Q;Blot, WJ;Wu, J;Johansson, M;Hultdin, J;Grankvist, K;Stevens, VL;McCullough, MM;Weinstein, SJ;Albanes, D;Ziegler, R;Freedman, ND;Langhammer, A;Hveem, K;Næss, M;Sesso, HD;Gaziano, JM;Buring, JE;Lee, IM;Severi, G;Zhang, X;Stampfer, MJ;Han, J;Smith-Warner, SA;Zeleniuch-Jacquotte, A;Le Marchand, L;Yuan, JM;Wang, R;Butler, LM;Koh, WP;Gao, YT;Rothman, N;Ericson, U;Sonestedt, E;Visvanathan, K;Jones, MR;Relton, C;Brennan, P;Johansson, M;Ulvik, A

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Impaired functional vitamin B6 status is associated with increased risk of lung cancer

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	<p>Stevens, Victoria; American Cancer Society, McCullough, Marjorie; American Cancer Society, Epidemiology and Surveillance Research Department, Weinstein, Stephanie; National Cancer Institute, Division of Cancer Epidemiology and Genetics</p> <p>Albanes, Demetrius; National Cancer Institute, Division of Cancer Epidemiology and Genetics</p> <p>Ziegler, Regina; National Cancer Institute, Division of Cancer Epidemiology and Genetics</p> <p>Freedman, Neal; National Cancer Institute, Division of Cancer Epidemiology and Genetics</p> <p>Langhammer, Arnulf; Norwegian University of Science and Technology, HUNT Research Centre, Department of Public Health and Nursing, Faculty of Medicine and Health Science</p> <p>Hveem, Kristian; Norwegian University of Science and Technology, HUNT Research Centre, Department of Public Health and Nursing, Faculty of Medicine and Health Science</p> <p>Nass, Marit; Norwegian University of Science and Technology, HUNT Research Centre, Department of Public Health and Nursing, Faculty of Medicine and Health Science</p> <p>Sesso, Howard; Brigham and Women's Hospital Division of Preventive Medicine; Harvard T.H. Chan School of Public Health, Department of Epidemiology</p> <p>Buring, Julie; Brigham and Women's Hospital and Harvard Medical School, Dept of Ambulatory Care and Prevention; Harvard T.H. Chan School of Public Health, Department of Epidemiology</p> <p>Lee, I-Min; Harvard School of Public Health, Department of Epidemiology; Harvard School of Public Health, Department of Epidemiology</p> <p>Gaziano, Michael; Brigham and Women's Hospital; VA Boston Healthcare System</p> <p>Severi, Gianluca; Human Genetics Foundation; COMUE Universite Paris-Saclay</p> <p>Zhang, Xuehong; Harvard Medical School, Medicine; Brigham and Women's Hospital,</p> <p>Stampfer, Meir; Brigham and Women's Hospital, ; Harvard Medical School, Medicine</p> <p>Han, Jiali; Harvard Medical School, Channing Laboratory, Medicine-Brigham and Women's Hospital</p> <p>Smith-Warner, Stephanie; Harvard T.H. Chan School of Public Health, Department of Epidemiology</p> <p>Zeleniuch-Jacquotte, Anne; New York University School of Medicine, Environmental Medicine</p> <p>Le Marchand, Loic; University of Hawaii Cancer Center, Epidemiology Program</p> <p>Yuan, Jian-Min; University of Pittsburgh, Epidemiology</p> <p>Wang, Renwei; University of Pittsburgh, Epidemiology</p> <p>Butler, Lesley ; University of Pittsburgh, Epidemiology</p> <p>Koh, Woon-Puay; National University of Singapore, Department of Epidemiology and Public Health and Family Medicine</p> <p>Gao, Yu-Tang; Shanghai Jiaotong University - Shanghai Cancer Institute, Department of Epidemiology</p> <p>Rothman, Nathaniel; National Cancer Institute, Div of Cancer Epidemiology & Genetics;</p> <p>Ericson, Urlika; Lund University, Department of clinical sciences</p> <p>Sonestedt, Emily; Lunds Universitet, Department of clinical sciences</p> <p>Visvanathan, Kala; 34. Johns Hopkins Bloomberg School of Public Health and Johns Hopkins Sidney Kimmel Comprehensive Center, School of Medicine</p> <p>Jones, Miranda ; 34. Johns Hopkins Bloomberg School of Public Health and Johns Hopkins Sidney Kimmel Comprehensive Center, School of Medicine</p> <p>Relton, Caroline; University of Bristol, Epigenetic epidemiology ; Newcastle</p>
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1 Impaired functional vitamin B6 status is associated with increased
2 risk of lung cancer

3
4 Research Article

5
6 **Authors**

7 Despoina Theofylaktopoulou¹, Øivind Midttun^{2,*}, Per M. Ueland^{1,3}, Klaus
8 Meyer², Anouar Fanidi^{4,5}, Wei Zheng⁶, Xiao-Ou Shu⁶, Yong-Bing Xiang^{6,7},
9 Ross Prentice⁸, Mary Pettinger⁸, Cynthia A. Thomson⁹, Graham G Giles^{11,12},
10 Allison Hodge^{11,12}, Qiuyin Cai⁶, William J. Blot⁶, Jie Wu⁶, Mikael Johansson¹²,
11 Johan Hultdin¹³, Kjell Grankvist¹³, Victoria L. Stevens¹⁴, Marjorie M.
12 McCullough¹⁴, Stephanie J. Weinstein¹⁵, Demetrius Albanes¹⁵, Regina
13 Ziegler¹⁵, Neal D. Freedman¹⁵, Arnulf Langhammer¹⁶, Kristian Hveem¹⁶, Marit
14 Næss¹⁶, Howard D. Sesso^{17,18,19}, J. Michael Gaziano¹⁸, Julie E. Buring^{17,19}, I-
15 Min Lee^{17,19}, Gianluca Severi^{21,22}, Xuehong Zhang²³, Meir J. Stampfer^{23,24,25},
16 Jiali Han²⁴, Stephanie A. Smith-Warner^{24,25}, Anne Zeleniuch-Jacquotte²⁶, Loic
17 le Marchand²⁷, Jian-Min Yuan^{28,29}, Renwei Wang²⁸, Lesley M. Butler^{28,29},
18 Woon-Puay Koh³⁰, Yu-Tang Gao³¹, Nathaniel Rothman³², Ulrika Ericson³³,
19 Emily Sonestedt³³, Kala Visvanathan³⁴, Miranda R. Jones³⁴, Caroline
20 Relton^{35,36}, Paul Brennan⁴, Mattias Johansson⁴, Arve Ulvik²

21 * Corresponding author:

22 email: Bjorn.Midttun@uib.no, phone: +47 55 97 46 04.

23 Adress: Postboks 7804, 5020 Bergen Norway

24

- 25 1. Department of Clinical Science, University of Bergen, Norway
26 2. Bevital AS, Bergen, Norway
27 3. Laboratory of Clinical Biochemistry, Haukeland University Hospital,
28 Bergen, Norway
29 4. Genetic Epidemiology Group, International Agency for Research on
30 Cancer, Lyon, France
31 5. MRC Epidemiology Unit, University of Cambridge, Cambridge, United
32 Kingdom
33 6. Division of Epidemiology, Department of Medicine, Vanderbilt
34 Epidemiology Center, Vanderbilt-Ingram Cancer Center, Vanderbilt
35 University School of Medicine, Nashville, USA
36 7. Department of Epidemiology, Shanghai Cancer Institute, Renji
37 Hospital, Shanghai Jiaotong University School of Medicine, Shanghai,
38 China
39 8. Division of Public Health Sciences, Fred Hutchinson Cancer research
40 Center, Seattle, USA

- 41 9. Health Promotion Sciences, Mel and Enid Zuckerman College of Public
42 Health, University of Arizona, Tucson, Arizona, USA
- 43 10. Cancer Epidemiology Centre, Cancer Council Victoria, Melbourne,
44 Victoria, Australia
- 45 11. Centre for Epidemiology and Biostatistics, Melbourne School of
46 Population and Global Health, University of Melbourne, Victoria,
47 Australia
- 48 12. Department of Radiation Sciences, Oncology, Umeå University, Umeå,
49 Sweden
- 50 13. Department of Medical Biosciences, Umeå University, Umeå, Sweden
- 51 14. Epidemiology Research Program, American Cancer Society, Atlanta,
52 GA, USA
- 53 15. Division of Cancer Epidemiology and Genetics, National Cancer
54 Institute, National Institutes of Health, Bethesda, Maryland, USA
- 55 16. HUNT Research Centre, Department of Public Health and Nursing,
56 Faculty of Medicine and Health Science, NTNU, Norwegian University
57 of Science and Technology, Trondheim, Norway
- 58 17. Division of Preventive Medicine, Brigham and Women's Hospital,
59 Boston, MA, USA
- 60 18. Division of Aging, Brigham and Women's Hospital, Boston, MA USA
- 61 19. Department of Epidemiology, Harvard T.H. Chan School of Public
62 Health, Boston, MA, USA
- 63 20. VA Boston Healthcare System, Boston, MA USA
- 64 21. Human Genetics Foundation (HuGeF), Torino, Italy
- 65 22. CESP (U1018 INSERM), Facultés de médecine Université Paris-Sud,
66 UVSQ, Université Paris-Saclay, Villejuif, France
- 67 23. Channing Division of Network Medicine, Department of Medicine,
68 Brigham and Women's Hospital and Harvard Medical School, Boston,
69 MA, USA
- 70 24. Department of Epidemiology, Harvard T. H. Chan School of Public
71 Health, Boston, MA, USA
- 72 25. Department of Nutrition, Harvard T.H. Chan School of Public Health,
73 Boston, MA, USA
- 74 26. Department of Population Health, New York University School of
75 Medicine, USA
- 76 27. Epidemiology Program, University of Hawaii Cancer Center, Honolulu,
77 HI, USA
- 78 28. Division of Cancer Control and Population Sciences, University of
79 Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania, USA
- 80 29. Department of Epidemiology, Graduate School of Public Health,
81 University of Pittsburgh, Pittsburgh, Pennsylvania, USA
- 82 30. Duke-NUS Medical School, Singapore and Saw Swee Hock School of
83 Public Health, National University of Singapore, Singapore
- 84 31. Department of Epidemiology, Shanghai Cancer Institute, Shanghai
85 Jiaotong University, Shanghai, China
- 86 32. Division of Cancer Epidemiology & Genetics, Occupational and
87 Environmental Epidemiology Branch, National Cancer Institute;
88 Rockville, USA
- 89 33. Department of clinical sciences Malmö, Lund University, Sweden

- 90 34. Johns Hopkins Bloomberg School of Public Health and Johns Hopkins
91 Sidney Kimmel Comprehensive Center, School of Medicine, USA
92 35. Institute of Genetic Medicine, Newcastle University, Newcastle, UK
93 36. MRC Integrative Epidemiology Unit, School of Social & Community
94 Medicine, University of Bristol, Bristol, UK
95

96 **Abbreviations**

- 97 PLP pyridoxal 5'-phosphate
98 HK:XA 3-hydroxykynurenine:xanthurenic acid
99 CI confidence interval
100 EPIC European Prospective Investigation into Cancer and Nutrition
101 ATBC Alpha-Tocopherol, Beta-Carotene Cancer Prevention
102 OR odds ratio
103 LC3 Lung Cancer Cohort Consortium
104

105 **Keywords:** Pyridoxal 5'-phosphate; Functional vitamin B6 marker ; 3-

106 hydroxykynurenine:xanthurenic acid; Lung cancer cohort consortium

107 **Novelty and Impact**

108 Low vitamin B6 status, assessed by circulating pyridoxal 5'-phosphate (PLP),
109 has been associated with increased risk of lung cancer. However, factors
110 other than vitamin B6 status may contribute to lower PLP, possibly
111 confounding its association with lung cancer. In the present study we
112 demonstrated, by using a novel functional biomarker of B6 status that
113 impaired functional vitamin B6 status was associated with increased risk of
114 lung cancer, especially squamous cell carcinoma.

115

116 **Abstract**

117

118 Circulating vitamin B6 levels have been found to be inversely associated with
119 lung cancer. Most studies have focused on the B6 form pyridoxal 5'-
120 phosphate (PLP), a direct biomarker influenced by inflammation and other
121 factors. Using a functional B6 marker allows further investigation of the
122 potential role of vitamin B6 status in the pathogenesis of lung cancer. We
123 prospectively evaluated the association of the functional marker of vitamin B6
124 status, the 3-hydroxykynurenine:xanthurenic acid ratio (HK:XA), with risk of
125 lung cancer in a nested case-control study consisting of 5,364 matched case
126 control pairs from the Lung Cancer Cohort Consortium (LC3). We used
127 conditional logistic regression to evaluate the association between HK:XA and
128 lung cancer, and random effect models to combine results from different
129 cohorts and regions. High levels of HK:XA, indicating impaired functional B6
130 status, were associated with an increased risk of lung cancer, the odds ratio
131 comparing the fourth and the first quartiles (OR_{4th vs 1st}) was 1.25 [95%
132 confidence interval, 1.10-1.41]. Stratified analyses indicated that this
133 association was primarily driven by cases diagnosed with squamous cell
134 carcinoma. Notably, the risk associated with HK:XA was approximately 50%
135 higher in groups with a high relative frequency of squamous cell carcinoma,
136 i.e. men, former and current smokers. This risk of squamous cell carcinoma
137 was present in both men and women regardless of smoking status.

138

139
140

141 **Introduction**

142 Lung cancer is the most common cause of cancer related death,
143 contributing to almost 20% of all cancer deaths worldwide ¹. The four major
144 histological types of lung cancer are adenocarcinomas, squamous cell
145 carcinomas, large cell carcinomas, and small cell carcinomas. The most
146 important risk factor for lung cancer is smoking, but the strength of the
147 association depends on the type of lung cancer ². Some lung cancer types
148 like small cell and squamous cell carcinomas occur almost exclusively due
149 to smoking, while others, like adenocarcinomas, also occur frequently in
150 non-smokers ².

151 Vitamin B6 may play a role in carcinogenesis, since it is involved in
152 DNA synthesis, methylation, and repair ³, chromosomal stability ⁴ and
153 oxidative stress ⁵. Indeed, circulating B6 measured as pyridoxal 5'-
154 phosphate (PLP) was found to be inversely associated with lung cancer risk
155 in two earlier case control studies, nested in prospective cohorts ^{6 7}, but in a
156 recent analysis within the Lung Cancer Cohort Consortium (LC3), vitamin
157 B6 was found to be only marginally associated with cancer risk in former
158 and current smoking men ⁸.

159 However, circulating levels of the vitamin B6 measure used in these
160 papers, the widely used PLP, are influenced by factors other than vitamin
161 B6 status. These factors include inflammation, alkaline phosphatase
162 activity, low serum albumin and renal function ⁹, and reduce the usefulness
163 of PLP as a marker of vitamin B6 status.

164 A recently established functional marker of vitamin B6 status is the
165 ratio of circulating levels of two metabolites in the kynurenine pathway of
166 tryptophan metabolism, 3-hydroxykynurenine (HK) and xanthurenic acid
167 (XA), i.e. HK:XA [4]. The conversion of HK to XA is catalyzed by the PLP-
168 dependent enzyme kynurenine aminotransferase, while the formation of HK
169 does not require PLP¹⁰. The substrate-product ratio HK:XA has been
170 shown to increase in B6 deficient individuals and reduced to normal levels
171 after supplementation with B6¹⁰.

172 Given the drawbacks of PLP as a marker of vitamin B6 status, the aim
173 of the present study was to use the functional vitamin B6 marker HK:XA to
174 further investigate the role of vitamin B6 status as a predictor of lung cancer
175 risk. The study used data from over 5,000 cases-controls pairs from the Lung
176 Cancer Cohort Consortium (LC3), nested within 20 prospective cohorts from
177 the USA, Europe, Asia and Australia.

178

179 **Methods**

180 **Study population**

181 All prospective cohort studies within the National Cancer Institute (NCI)
182 Cancer Consortium were invited to participate in the study. Twenty cohorts,
183 from USA (11 cohorts), Europe (total of 4 cohorts from Norway, Sweden, and
184 Finland), Asia (4 cohorts consisting of Chinese populations residing in
185 Shanghai and Singapore) and Australia (1 cohort), fulfilled the inclusion
186 criteria (having cryopreserved baseline plasma or serum samples, and being
187 members of the US National Cancer Institute (NCI) Cohort Consortium in

188 2009) and accepted to participate. Details on design of the cohorts and their
189 follow-up procedures have been previously published ⁸.

190

191 **Selection of cases and controls**

192 Lung cancer cases were defined on the basis of the International
193 Classification of Diseases for Oncology, Second Edition (ICD-O-2) and
194 included invasive cancers coded as C34.0-C34.9. From the 11,399 incident
195 lung cancer cases with pre-diagnostic blood samples, 5,545 cases were
196 selected by oversampling never and former smoking cases. For each case,
197 one control was randomly chosen from risk-sets consisting of all cohort
198 members alive and free of cancer (except non-melanoma skin cancer) at the
199 time of diagnosis of the index case. Matching criteria were cohort, sex, date of
200 blood collection, and date of birth. Controls were also matched by smoking
201 status at time of blood collection in 5 categories; never smokers, short and
202 long term quitters among former smokers (<10 years, ≥10 years since
203 quitting), and light and heavy smokers among current smokers (< 15, ≥15
204 cigarettes per day). In total, 5,364 lung cancer case-control pairs were eligible
205 for inclusion after excluding cases who were not correctly matched on
206 smoking status (n=124), who had insufficient plasma sample volume for
207 analysis of biomarkers (n=42), or had a revised date of diagnosis prior to
208 blood draw (n=13) ⁸.

209

210 **Biochemical analyses**

211 Analysis of all serum or plasma samples was performed in the Bevital
212 A/S laboratory (<http://www.bevital.no>) in Bergen, Norway. Concentrations of

213 HK, XA, PLP and cotinine, a marker of recent nicotine exposure ¹¹ were
214 determined using a liquid chromatography–tandem MS assay ¹², and C-
215 reactive protein (CRP) was analysed by immuno-MALDI-MS ¹³ in batches of
216 86 samples. Quality control procedures included 6 calibration plasma, 2
217 control plasma, and 1 blank sample (water) in each batch. All blood samples
218 were stored at -80°C or lower until analysis and cases and their matched
219 controls were analyzed together within the same batches in random order,
220 with laboratory staff blinded to case-control status. Further details on the
221 biochemical analyses have been published elsewhere ⁸.

222

223 **Statistical analysis**

224 We used conditional logistic regression (conditioning on individual case
225 sets) to calculate the odds ratios (OR) with 95% confidence intervals (CI) for
226 lung cancer according to levels of HK:XA. The analysis was adjusted for
227 smoking intensity using quartiles of cotinine concentrations based on the
228 distribution of cotinine among current smokers. We performed analyses within
229 each cohort, comparing the fourth to the first quartile (OR_{4th} vs_{1st}) of HK:XA.
230 Results were combined for each region (United States [USA], Europe, Asia,
231 Australia), and for the overall study population by using random effects
232 models. Heterogeneity across subgroups was quantitatively assessed by the
233 Q-test and I^2 index¹⁴.

234 We further performed stratified analyses for sex, smoking category
235 (never, former, and current smokers), histology of lung cancer (by HK:XA
236 tertiles), and time between blood sample collection and diagnosis. Due to the
237 large differences in vitamin status between regions¹⁵, quartiles (or tertiles) of

238 concentrations for each biomarker were based on the distribution among
239 controls by region. We additionally used conditional logistic regression for
240 calculating the odds ratio for lung cancer across quartiles by region and for
241 the total population, using the first quartile as reference. Quartiles were
242 included as a continuous variable to calculate p for trend.

243 In supplementary analysis stratified by histology in addition to smoking
244 status or sex we included HK:XA as a continuous variable, using the base-2
245 logarithm (\log_2) of the biomarker in a conditional logistic regression model.
246 Estimates from this model may be interpreted as the relative risk associated
247 with a doubling in circulating biomarker concentration. Partial Spearman
248 correlations adjusted for age and sex were used to describe the association
249 between HK:XA and PLP, and both biomarkers with cotinine. All statistical
250 analyses were conducted using R 3.2.2 for Macintosh¹⁶. The package
251 “survival”¹⁷ was used for conditional logistic regression, and package
252 “metafor” for forestplots¹⁸.

253

254 **Results**

255 **Study population**

256 The final study population included 5,364 lung cancer cases and 5,364
257 matched controls, with a median age of 62 years at blood sample collection
258 (Table 1). Median time between blood draw and lung cancer diagnosis was
259 6.3 years. Of the total study population, 46% of the participants were women.
260 At baseline, nearly half of the participants were current smokers, and one
261 fourth were former, and never smokers, respectively (Table 1). Due to
262 different inclusion criteria in the original cohorts, five cohorts (Health

263 Professionals Follow-up Study, Physicians Health Study, ATBC, The
264 Shanghai Cohort Study and The Shanghai Mens' Health Study) included only
265 men, and five cohorts (WHI, NYUWHS, WHS, NHS and SWHS) only women
266 (Figure 1). The prevalence of smoking also differed substantially between
267 cohorts (Figure 1).

268

269 **Determinants of HK:XA within the LC3**

270 HK:XA varied somewhat across regions, (median values ranging from
271 2.88 to 3.28 among controls) with the lowest level among Australian controls
272 and the highest among Europeans. Larger variations were observed for
273 plasma PLP, with the highest concentrations in the controls from US cohorts
274 (median 49.9 nmol/L) and the lowest concentrations among the European
275 cases, at 28.1 nmol/L. We observed an inverse relation between HK:XA and
276 PLP (Spearman rho =-0.37), while smoking was essentially not associated
277 with HK:XA (rho =0.11), but was inversely related to plasma PLP (rho =-0.30)
278 (all $p < 0.001$).

279

280 **HK:XA and lung cancer**

281 Random effects models were used to investigate the relation of HK:XA
282 with risk of lung cancer across geographic regions because the heterogeneity
283 by cohort varied significantly across the geographic regions (Supplemental
284 Table S1). Overall, high levels of HK:XA (4th vs. 1st quartile) were associated
285 with a 25% increased risk for lung cancer (Figure 2). However, results differed
286 across regions with positive associations observed in Europe, with an odds
287 ratio comparing the fourth and the first quartiles (95% confidence interval) of

288 1.43 (1.06, 1.95), and the USA 1.31 (1.05, 1.62), but no association in Asia or
289 Australia (Figure 2). Results were similar when using quartiles based on the
290 distribution of each region, instead of cohort specific cut-offs (Supplemental
291 Table S2).

292 The weakest associations were observed in cohorts that included only
293 women. When those cohorts (The Women's Health Initiative, The New York
294 University Women's Health Study, Women's Health Study and Nurses Health
295 Study) were excluded, the association of HK:XA with risk of lung cancer in the
296 USA was similar, 1.41 (1.15, 1.46), to that seen in Europe. Additional
297 adjustment for CRP, a marker for systemic inflammation, did not attenuate the
298 risk association for HK:XA (data not shown).

299

300 **Analyses stratified by sex and smoking**

301 In analysis stratified by sex the overall association between HK:XA and
302 lung cancer risk was primarily seen among men, with a 50% increased risk of
303 lung cancer when comparing fourth vs. first quartile (Figure 3). No significant
304 association was observed for women, (p heterogeneity = 0.01 and $I^2 = 62.4\%$,
305 Supplemental Table S3). Smoking habits differed between sexes, with the
306 proportion of never smokers much higher among women (Figure 1). A similar
307 effect modification was present for smoking categories, with the association
308 between HK:XA and lung cancer limited to current and former smokers (p for
309 heterogeneity = 0.18, $I^2 = 30.1\%$, Supplemental Table S4) (Figure 4).

310

311 **Histology of lung cancer**

312 Histology of lung cancer differed according to smoking status, with
313 squamous cell carcinoma being more common among current and former
314 smokers (28% and 20% respectively, compared to 6% among never smokers)
315 and in men compared to women (29% vs. 10%). In analysis according to
316 histology of lung cancer in the overall population, HK:XA was related to an
317 increased risk for squamous cell carcinoma OR (95%CI) 1.42 (1.10, 1.82) for
318 3rd vs. 1st tertile, but not with other histological types (data not shown).

319 This association with HK:XA and squamous cell carcinoma was
320 consistently present in subgroup analysis by both sex and smoking status.
321 Specifically, for a continuous log₂ model, representing a doubling of HK:XA
322 concentrations, the OR (95%CI) was 1.20 (1.02, 1.41) in men, 1.59 (1.20,
323 2.10) in women (Supplemental Figure S2). In current smokers the OR (95%
324 CI) was 1.22 (1.02, 1.46), in former smokers 1.37 (1.08, 1.73), and in never
325 smokers 1.59 (0.90, 2.80), even though in this last group the confidence
326 interval was quite wide due to the low number of cases (Supplemental figure
327 S1).

328

329 **Time to diagnosis**

330 In analysis stratified by time to diagnosis, the association was limited to
331 participants who were diagnosed with lung cancer within 36 months from
332 blood draw, OR_{log₂} (95%CI) 1.43 (1.27, 1.61) for a doubling in the
333 concentration of HK:XA. No significant association between HK:XA and lung
334 cancer risk was observed for those with a longer time between blood draw
335 and diagnosis (p for heterogeneity <0.001).

336

337 **Discussion**

338 **Main findings**

339 High levels of HK:XA, indicating an impaired functional vitamin B6
340 status, were associated with an increased risk of lung cancer. In stratified
341 analysis the risk of lung cancer was approximately 50% higher for those in the
342 highest category of HK:XA in men, and in former and current smokers, but not
343 significant in women or never smokers. In analysis stratified by histology
344 HK:XA was associated with an increased risk of squamous cell carcinoma,
345 but not other histological types. When histopathology subtype of lung cancer
346 was considered, a consistent association was found for squamous cell
347 carcinoma regardless sex and smoking status. The lack of association of
348 HK:XA with overall lung cancer among women and never smokers could be at
349 least partly attributed to the low number of cases of squamous cell carcinoma
350 in those strata of the present study population.

351

352 **Comparison with previous findings**

353 Overall, our findings are in agreement with published results on the B6
354 vitamers PLP and cancer risk ¹⁹, even though stronger inverse associations
355 were noted in relation to lung cancer in the EPIC ⁶ and ATBC ⁷ studies.
356 Concordant with the current study, we recently observed an inverse
357 association of PLP with lung cancer risk in LC3, an association that was
358 primarily confined to former and current smoking men ⁸.

359 We observed a positive association between HK:XA and risk of
360 squamous cell carcinoma, but no significant association with other histological
361 types of lung cancer. This observation is also in line with a previous

362 observation of an inverse association between plasma PLP and risk of cancer
363 primarily classified as squamous cell carcinoma⁸.

364 In EPIC an inverse association of PLP on lung cancer was also
365 observed in never smokers, but the number of cases that were never smokers
366 was low (n=96)⁶, and this results should be viewed with caution.

367 In a previous cohort study where PLP and HK:XA were
368 simultaneously assessed as predictors of cancer no clear association was
369 found for any of the two markers. However, this study had limited statistical
370 power due to the small number of cases (n_{cases}=85)²⁰.

371

372 **HK:XA as a marker of vitamin B6 status and predictor of lung cancer**

373 There are consistent reports on plasma PLP as a predictor of cancer in
374 the lungs^{6,7} and other organs¹⁹. Plasma PLP is the most commonly used
375 marker of vitamin B6 status, but plasma PLP concentrations are reduced by
376 several factors linked to lung cancer carcinogenesis or progression, such as
377 smoking²¹, inflammation measured as CRP²²⁻²⁴, and increased level of
378 alkaline phosphatase²⁵. On the other hand, inflammation and elevated
379 alkaline phosphatase (ALP) are not associated with impaired vitamin B6
380 availability in tissues⁹.

381 Smoking is associated with lower levels of PLP, and vitamin B6 status
382 gradually improves over years after smoking cessation²⁶. In contrast,
383 smoking shows no or a weak association with the HK:XA ratio¹⁰, an
384 observation confirmed in the present study. In the current study, cases and
385 controls were matched for smoking status and we additionally adjusted for
386 smoking intensity, using circulating cotinine concentrations. We cannot

387 exclude residual confounding by smoking, but since the association between
388 HK:XA and lung cancer was also present in former smokers, confounding by
389 smoking is unlikely.

390 CRP is inversely associated with plasma PLP^{27, 28} but shows a weak
391 positive association with HK:XA¹⁰. After additional adjustment for CRP, the
392 risk estimates of HK:XA and lung cancer remained essentially the same,
393 suggesting no or minor confounding from inflammation. Elevated ALP may
394 reduce PLP through conversion to pyridoxal (PL)⁹, but HK and XA are not
395 substrates for ALP, and one would not expect any direct effects from ALP on
396 the plasma levels of these metabolites.

397 Similarly to the findings on PLP in the LC3 study⁸, the association
398 between HK:XA and lung cancer was stronger among participants with a short
399 time between blood draw and diagnoses.

400 Therefore, it is possible that the observed association between HK:XA
401 and risk may reflect impaired vitamin B6 status due to pre-clinical changes in
402 lung cancer.

403

404 **Strengths and limitations of the study**

405 The present study is based on a an unprecedented sample of 5,364
406 pre-diagnostic blood samples from lung cancer cases with comparable control
407 samples recruited in 20 prospective cohorts from around the world. The
408 prospective study design minimizes the risk of reverse causality and selection
409 bias. The use of a centralized laboratory with a stringent quality control
410 protocols and cases and matched controls analyzed together minimizes any
411 technical differential bias, and an overrepresentation of never and former

412 smokers provided adequate power for stratified analysis. By using a functional
413 marker that is largely independent on factors that are related to circulating
414 PLP, we found a clear inverse relation of vitamin B6 status with risk of lung
415 cancer.

416 There was only one blood sample available for measurement of
417 biomarkers for each participant, so the association between HK:XA and lung
418 cancer may be attenuated due to regression dilution bias. It is possible that
419 depending on the time of the blood draw and the length of study follow-up, the
420 single measurement may not represent the exposure period most relevant for
421 lung cancer development. Lastly, information on the histology of lung cancer
422 was missing for 34 % of the participants.

423

424 **Conclusions**

425 Our findings provide evidence for an inverse association of functional
426 vitamin B6 status and risk of lung cancer, especially squamous cell
427 carcinoma. This expands our understanding beyond what can be concluded
428 from the modest relation observed for the direct vitamin B6 marker PLP ⁸,
429 circulating levels of which is influenced by factors other than vitamin B6
430 status, in this same study. It is recommended that future studies strive for a
431 sample size large enough to provide the power necessary for analysis
432 stratified by duration of follow-up, smoking and histology given the potential
433 differences of the role of vitamin B6 in pathogenesis and progression of
434 different histological cancer types.

435

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437
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468

469 **Competing interests**

470 LMB is an employee of Genetech Inc. as of September 16.

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473

474 **References**

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Table 1. Baseline and clinical characteristics of study participants overall and according to region¹

Characteristics	Overall		Asian cohorts		Australian cohort		European cohorts		USA cohorts	
	Controls (n=5364)	Cases (n=5364)	Controls (n=1775)	Cases (n=1775)	Controls (n=354)	Cases (n=354)	Controls (n=835)	Cases (n=835)	Controls (n=2400)	Cases (n=2400)
Age ² (years)	62 (47-75)	62 (47-75)	62 (46-74)	62 (46-74)	61 (45-67)	61 (45-67)	60 (45-71)	60 (45-71)	64 (48-78)	64 (48-78)
Sex										
Men	2908 (54%)	2908 (54%)	1229 (69%)	1229 (69%)	213 (60%)	213 (60%)	475 (57%)	475 (57%)	991 (41%)	991 (41%)
Women	2456 (46%)	2456 (46%)	546 (31%)	546 (31%)	141 (40%)	141 (40%)	360 (43%)	360 (43%)	1409 (59%)	1409 (59%)
Smoker										
Never	1327 (25%)	1327 (25%)	602 (34%)	602 (34%)	49 (14%)	49 (14%)	107 (13%)	107 (13%)	569 (24%)	569 (24%)
Former	1518 (28%)	1518 (28%)	176 (10%)	176 (10%)	145 (41%)	145 (41%)	190 (23%)	190 (23%)	1007 (42%)	1007 (42%)
Current	2519 (47%)	2519 (47%)	997 (56%)	997 (56%)	160 (45%)	160 (45%)	538 (64%)	538 (64%)	824 (34%)	824 (34%)
Biomarkers										
HK:XA	2.98 (1.39-7.70)	3.13 (1.44-8.49)	3.01 (1.52-7.09)	3.10 (1.58-7.98)	2.88 (1.45-6.68)	3.00 (1.34-7.55)	3.08 (1.58-7.08)	3.28(1.64-9.13)	2.93 (1.27-8.24)	3.13 (1.29-8.98)
HK (nmol/L)	36.6 (20.3-70.6)	37.1 (20.1-74.5)	38.7 (21.9-81.6)	39.6 (22.4 -85.4)	36.0 (22.1-65.8)	37.8 (21.3-69.3)	37.2 (21.6-63.9)	38.3 (23.1-67.2)	34.8 (18.9-65.2)	34.7 (18.2-66.2)
XA (nmol/L)	12.4 (4.6-29.1)	11.9 (4.3-28.7)	13.5 (5.4-29.9)	13.0 (5.1 -29.2)	12.4 (4.9-26.5)	12.9 (5.2-29.4)	11.9 (5.1-26.5)	11.4 (4.5-27.1)	11.8 (4.2-29.4)	11.1 (3.9-29.1)
PLP (nmol/L)	37.1 (13.9-197)	35.1 (12.5-204)	30.8 (12.3-118)	28.9 (11.0-114)	31.3 (14.3-110)	31.3 (14.2-207)	30.9 (13.1-101)	28.1 (12.5-104)	49.9 (16.4-271)	47.6 (15.2-266)
Clinical characteristics										
Age at diagnosis (years)		69.8 (53.6-82.0)		69 (52-80)		70 (56-78)		68 (53-81)		70 (55-83)
Time to diagnosis ³ (years)		6.3 (1.0-16.0)		5.8 (0.7-16.5)		9.7 (1.3-16.2)		10.0 (1.8-16.1)		5.2 (1-15.5)
Histology										
Large cell carcinoma		174 (3%)		16 (1%)		31 (9%)		15 (2%)		112 (5%)
Small cell carcinoma		492 (9%)		99 (6%)		47 (13%)		103 (12%)		245 (10%)
Squamous cell carcinoma		836 (16%)		319 (18%)		67 (19%)		162 (19%)		291 (12%)
Adenocarcinoma		2056 (39%)		615 (35%)		153 (43%)		260 (31%)		1034 (43%)
Missing / Unknown		1806 (34%)		726 (41%)		56 (16%)		295 (19%)		735 (31%)

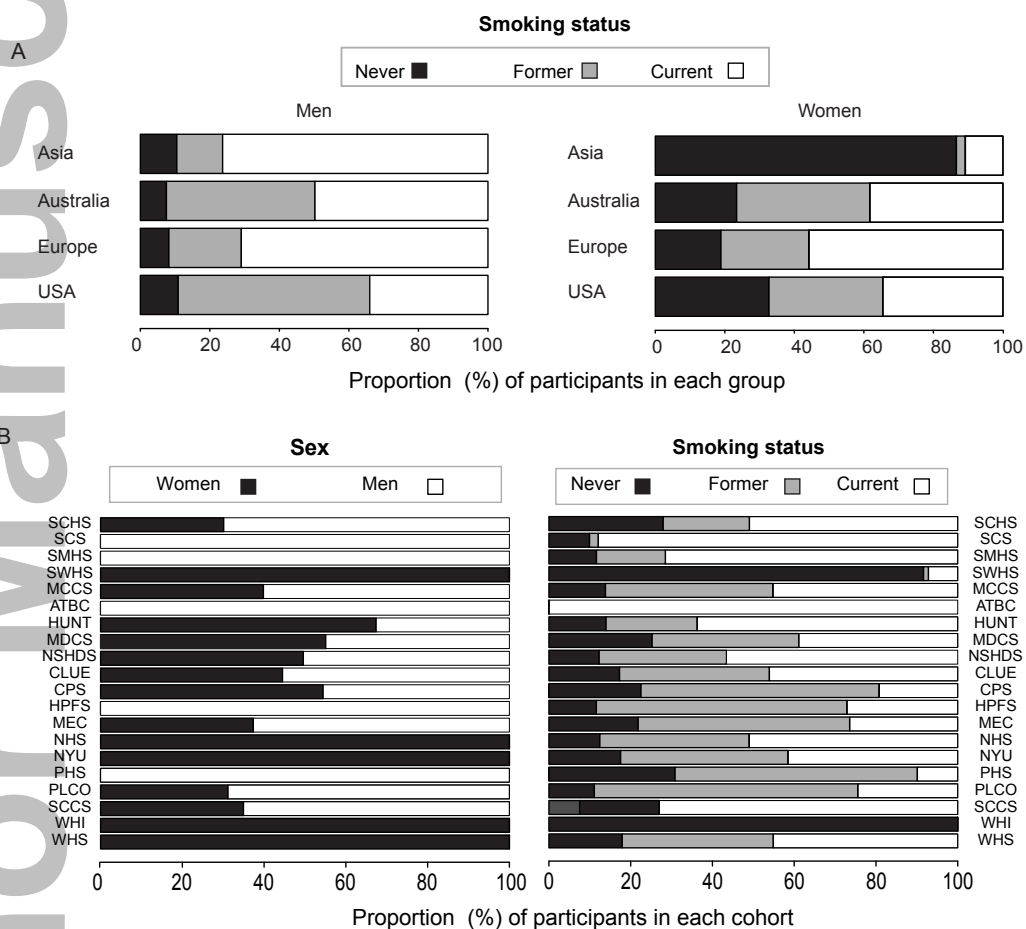
¹ Characteristics are presented as n (%) for discrete variables and median (5th, 95th percentile) for continuous variables

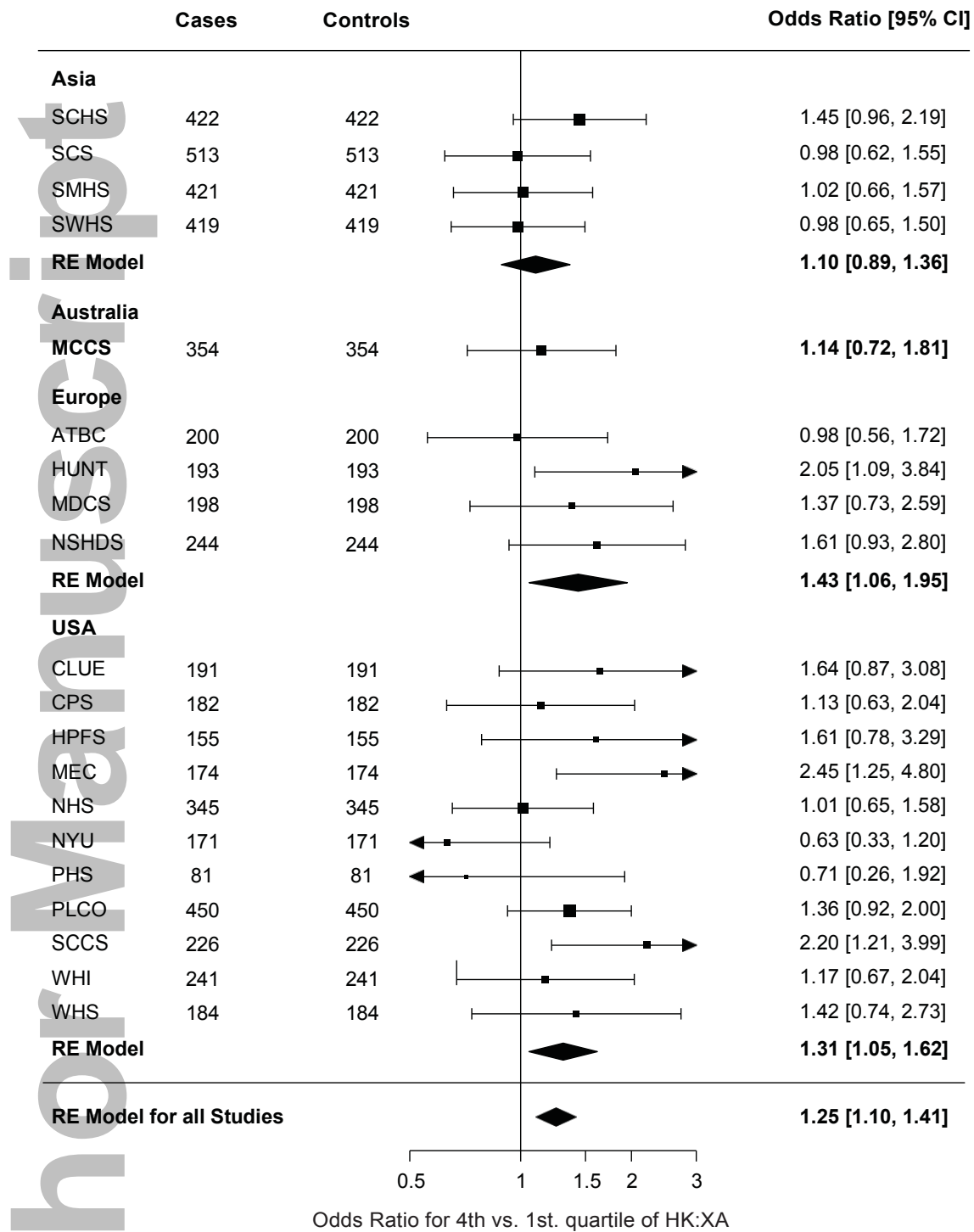
² At blood collection

³ Time from blood draw to diagnosis

HK:XA, 3-hydroxykynurenine:xanthurenic acid; PLP, pyridoxal 5'-phosphate

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