Serum B Vitamin Levels and Risk of Lung Cancer

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Context B vitamins and factors related to 1-carbon metabolism help to maintain DNA integrity and regulate gene expression and may affect cancer risk.

Objective To investigate if 1-carbon metabolism factors are associated with onset of lung cancer.

Design, Setting, and Participants The European Prospective Investigation into Cancer and Nutrition (EPIC) recruited 519 978 participants from 10 countries between 1992 and 2000, of whom 385 747 donated blood. By 2006, 899 lung cancer cases were identified and 1770 control participants were individually matched by country, sex, date of birth, and date of blood collection. Serum levels were measured for 6 factors of 1-carbon metabolism and cotinine.

Main Outcome Measure Odds ratios (ORs) of lung cancer by serum levels of 4 B vitamins (B_2, B_6, folate [B_9], and B_{12}), methionine, and homocysteine.

Results Within the entire EPIC cohort, the age-standardized incidence rates of lung cancer (standardized to the world population, aged 35-79 years) were 6.6, 44.9, and 156.1 per 100 000 person-years among never, former, and current smokers for men, respectively. The corresponding incidence rates for women were 7.1, 23.9, and 100.9 per 100 000 person-years, respectively. After accounting for smoking, a lower risk for lung cancer was seen for elevated serum levels of B_6 (fourth vs first quartile OR, 0.44; 95% confidence interval [CI], 0.33-0.60; *P* for trend <.000001), as well as for serum methionine (fourth vs first quartile OR, 0.52; 95% CI, 0.39-0.69; *P* for trend <.000001). Similar and consistent decreases in risk were observed in never, former, and current smokers, indicating that results were not due to confounding by smoking. The magnitude of risk was also constant with increasing length of follow-up, indicating that the associations were not explained by preclinical disease. A lower risk was also seen for serum folate (fourth vs first quartile OR, 0.68; 95% CI, 0.51-0.90; *P* for trend = .001), although this was apparent only for former and current smokers. When participants were classified by median levels of serum methionine and B_6, having above-median levels of both was associated with a lower lung cancer risk overall (OR, 0.41; 95% CI, 0.31-0.54), as well as separately among never (OR, 0.36; 95% CI, 0.18-0.72), former (OR, 0.51; 95% CI, 0.34-0.76), and current smokers (OR, 0.42; 95% CI, 0.27-0.65).

Conclusion Serum levels of vitamin B_6 and methionine were inversely associated with risk of lung cancer.

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B VITAMINS, INCLUDING B_6 AND FOLATE (B_9), as well as related enzymes in the 1-carbon pathway, are essential for DNA synthesis and methylation. The 1-carbon metabolism process is complex and involves multiple interactions between B vitamins, homocysteine, and methionine, which in turn are required for generation of S-adenosyl methionine, an essential component of methylation reactions.[1] Deficiencies in B vitamins may increase the probability of DNA damage and subsequent gene mutations, and may influence gene expression via aberrant methylation patterns.[2] Given their involvement in maintaining DNA integrity and gene expression, these nutrients have a potentially important role in inhibiting cancer development, and offer the possibility of modifying cancer risk through dietary changes.

Major sources of 1-carbon nutrients and related vitamins are varied and include fruits and green leafy vegetables (folate), fortified cereals and whole grains (B_6), as well as meat and dairy products (B_{12}).[3,4] B vitamin levels are also likely to be influenced by genetic variants and other factors including alcohol consumption and low-grade inflammation.[5-9] Although many countries have initiated folic acid supplementation of flour and other foodtypes, deficiencies in nutrient levels of B vitamins have been shown to be high in many western populations.[10]

Until now, the main focus of studies of B vitamins and cancer prevention has been on folate and colorectal cancer. Two randomized trials of folate supplementation investigated whether it may prevent colorectal adenomas among high-risk populations, but failed to identify a protective effect.[11,12] Although randomized trials may restrict confounding from other exposures, they have limitations in assessing the role of specific nutrients because (1) they are limited in size and the number of cancers that occur in the follow-up period; (2) supplementation is randomized over a relatively short period (several years); and (3) they are unrelated to lifelong vitamin levels prior to the study.

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Alternatively, large population cohorts with baseline blood collection can compare vitamin serum levels with subsequent cancer development in large numbers. Validity of the results depends on several assumptions regarding the measurement of single serum markers at baseline including that they are (1) representative of past exposures, (2) not associated with underlying preclinical disease, and (3) not explained by other causes of the disease such as smoking. A potential role of B_6 in lung cancer has been reported from a randomized trial of α-tocopherol and beta carotene (the ATBC study) in 29 000 male smokers in Finland.[13] Interpretation of this study is difficult due to the limited sample size, the absence of never smokers, and the possibility that smoking may suppress B_6 levels.

We therefore conducted a comprehensive investigation of B vitamins and methionine status based on serum samples from the the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort study, a large population cohort of more than 500 000 adults conducted in 10 European countries.

METHODS

Study Cohort

EPIC recruitment procedures, collection of questionnaire data, anthropometric measurements, and blood samples have been described in detail elsewhere.[14] In brief, standardized questionnaire data on dietary and nondietary variables were collected between 1992 and 2000 from 519 978 individuals across Europe, of whom 385 747 provided a blood sample. The present study included individuals diagnosed with lung cancer after blood collection in the case group and matched participants in the control group from 8 of the 10 participating countries: France, Italy, Spain, United Kingdom, the Netherlands, Greece, Germany, and Sweden (excluding the Malmö center).

A blood sample was collected according to a standardized protocol. Filled syringes were kept at 5°C to 10°C, protected from light, and transferred to a local laboratory for further processing. Blood fractions (serum, citrate plasma, red cells, and buffy coat) were aliquoted into 0.5-mL straws that were subsequently heat sealed and stored in liquid nitrogen tanks at the International Agency for Research on Cancer (IARC), Lyon, France, at –196°C, except in Umeå, Sweden, where samples were stored in 1.8-mL plastic tubes in –80°C freezers.

Follow-up for Cancer Incidence

In Italy, the Netherlands, Spain, Sweden, and Great Britain, incident cancer cases were identified through record linkage with regional or national cancer registries. In France, Germany, and Greece, follow-up was based on a combination of methods, including health insurance records, cancer and pathology registries, and active follow-up through study participants and their next of kin. For each EPIC study center, closure dates of the study period were defined as the latest dates of complete follow-up for both cancer incidence and vital status (dates varied between centers, December 2002-December 2005). Vital status follow-up was more than 98% complete.

Selection of Case and Control Group Participants

Among the 519 978 EPIC participants, 2206 were diagnosed with incident lung cancer by the end of the follow-up period for all centers. Individuals who did not donate a blood sample, had missing information on the date of blood donation, or had a history of another cancer (except nonmelanoma skin cancer) at the time of blood donation were excluded (n = 614), leaving 1592 case participants. After further exclusions of Norway (n = 15), Denmark (n = 473), and the Malmö center in Sweden (n = 202), serum samples (plasma in Umeå) were available for 899 case participants (3 did not have serum available). Data on histology were collected from each center where possible. Lung cancer cases were defined on the basis of the *International Classification of Diseases for Oncology, Second Edition*, and included all invasive cancers that were coded as C34.

For each case participant, 2 control participants were chosen at random from appropriate risk sets consisting of all cohort members alive and cancer free (except nonmelanoma skin cancer) at the time of diagnosis of the index case. Matching criteria were country, sex, date of blood collection (±1 month, relaxed to ±5 months for sets without available controls), and date of birth (±1 year, relaxed to ±5 years for sets without available control participants). Two control participants were available for 873 in the case group, and 1 control participant was available for 24 in the case group, resulting in a matched sample size of 897 case and 1770 control participants. No control participants were available for 2 in the case group, and a further 47 control participants were included from 1 center (Umeå) without a matched case participant. These 49 control participants do not contribute to subsequent overall matched analyses, although they were retained in the data set and contribute to unmatched stratified analysis. All participants gave written informed consent to participate in the study and the research was approved by the local ethics committees in the participating countries and the IARC institutional review board.

Biochemical Analyses

All biochemical analyses were performed at Bevital A/S (http://www.bevital.no), Bergen, Norway. The study included measurements of serum concentrations (plasma from Umeå) of B_2 (riboflavin), B_6 (measured as pyridoxal 5′-phosphate, its active form), folate (B_9), B_{12} (cobalamin), total homocysteine, and methionine. All case participants and all but 2 control participants were successfully analyzed. Along with pyridoxal 5′-phosphate, 2 other forms of B_6 were measured: pyridoxal, which is converted into pyridoxal 5′-phosphate, and pyridoxic acid, the catabolite of pyridoxal 5′-phosphate that is excreted in the urine. We also measured cotinine as an indicator of recent smoking behavior. Concentrations of B_2, B_6, homocysteine, methionine, and cotinine were determined by mass spectrometry–based methods (liquid chromatography coupled to tandem mass spectrometry; gas chromatography coupled to tandem mass spectrometry),[15,16] and microbiological methods were used to determine concentrations of folate (*Lactobacillus casei*)[17,18] and B_{12} (*Lactobacillus leichmannii*).[19]

Samples were analyzed in batches of 86 and quality control included 6 calibration samples, 2 control samples, and 1 blank sample in each batch. Samples from case and control participants were kept at –80°C and analyzed in random order. All staff in the Bevital laboratory were blinded to the case-control status of the blood samples.

Statistical Analyses

Age-standardized incidence rates per 100 000 person-years of lung cancer for the complete EPIC cohort of 519 978 individuals were calculated separately by sex and smoking status, and standardized to the world population aged 35 through 79 years.[20]

Overall risk analyses of lung cancer involved calculating quartiles of serum levels for each of the 4 B vitamins, as well as methionine and homocysteine, based on the distribution among control participants. The odds ratio (OR) and 95% confidence interval (CI) of lung cancer for participants in the second, third, and fourth quartile was calculated relative to the first quartile using conditional logistic regression, conditioning on individual case sets.

Additional adjustment was conducted for quartiles of cotinine level, which was considered to be the most accurate measure of smoking intensity at the time of blood collection. Including further smoking variables (smoking status, duration of smoking, average cigarettes smoked per day) did not alter the results notably. Additional adjustment was also conducted for body mass index (BMI [calculated as weight in kilograms divided by height in meters squared]), educational attainment, and alcohol consumption at the time of recruitment. Analyses were also conducted after stratifying for never, former, and current smokers using unconditional logistic regression adjusting for age at recruitment, sex, country, and in current smokers, quartiles of cotinine levels. The overall trend for each analyte (*P* for trend), as well as stratified analyses, were conducted by including the base 2 logarithm ($\log_2$) of the analyte concentrations as a continuous variable in a separate logistic regression model. The OR trend estimate from this model may be interpreted as the relative risk associated with a doubling in concentrations. All analyses were also repeated after removing case participants diagnosed within 1 year of blood collection.

Prespecified stratified analyses were conducted for country, histology, smoking status (including time since quitting among former smokers), sex, time from blood draw to diagnosis, as well as educational attainment and alcohol intake at recruitment. We used χ^2 tests to examine heterogeneity in OR in stratified analyses. Dietary intake of major food groups, as well as B_2, B_6, and B_{12}, were available as assessed by the EPIC food frequency questionnaires in each center. The association between lifestyle and dietary factors with serum levels were investigated using linear regression models, adjusting for case-control status, age, sex, and country, and further adjusted for cotinine when appropriate (in quartiles).

Cumulative risks of lung cancer were calculated up to the age of 79 years by estimating cumulative rates (the sum of age-specific incidence rates by sex and smoking status in 5-year categories) and applying a standard formula to convert these to cumulative risks.[20] These risks do not take into account competing causes of death. Similarly, cumulative risks by 1-carbon exposure categories were calculated by applying OR estimates and control exposure distributions on the cumulative rates.[21] These were calculated separately for men and women, and for never, former, and current smokers.

All *P* values were 2-sided and statistical analyses were conducted using SAS version 9.2 (Cary, North Carolina).

RESULTS

Incidence Rates of Lung Cancer Within the EPIC Cohort

Within the entire EPIC cohort of 519 978 individuals, the age-standardized incidence rates of lung cancer (standardized to the world population aged 35-79 years) were for men 6.6, 44.9, and 156.1 per 100 000 person-years among never, former, and current smokers, respectively. The corresponding incidence rates for women were 7.1, 23.9, and 100.9 per 100 000 person-years, respectively.

Baseline Characteristics of Case and Control Participants

Among the 899 case and 1815 control participants within the nested case-control study, 11% of case participants were never smokers and 29% were former smokers at the time of recruitment, compared with 39% and 37% of control participants, respectively (TABLE 1). Among both case and control participants, 62% were men and their median age at blood draw was 59 years (95% range, 43-73 years). The median time between blood draw and diagnosis of lung cancer among the case participants was 62 months (Table 1). Serum levels of B_2, B_6, folate, B_{12}, and methionine were similar between never and former smokers, although lower in current smokers (eTable 1 available at http://www.jama.com). Similarly, smoking intensity among current smokers (assessed by cotinine) was inversely associated with folate, B_{12}, and B_6.

Serum Levels of B Vitamins and Lung Cancer Risk

Case and control participants were subsequently compared for quartiles of serum levels of each of the four B vitamins, as well as homocysteine and methionine (TABLE 2). After adjusting for matching variables and cotinine, a substantial lower risk for lung cancer was seen for increasing levels of B_6 (fourth vs first quartile OR, 0.44; 95% CI, 0.33-0.60; *P* for trend $<.000001$). A lower risk was also seen for increasing methionine (fourth vs first quartile OR, 0.52; 95% CI, 0.39-0.69; *P* for trend $<.000001$). Moderate decreases in risk were seen for the second quartile of both B_6 and methionine (second vs first quartile OR, 0.78 [95% CI, 0.60-1.01] and 0.88 [95% CI, 0.69-1.15], respectively), as well as for the third quartile (third vs first quartile OR, 0.53 [95% CI, 0.40-0.71] and 0.49 [95% CI, 0.36-0.65], respectively). Adjustment by additional variables including BMI, educational attainment, and alcohol consumption did not modify the results (Table 2), and neither did simultaneous adjustment of each analyte (Table 2). Excluding case participants who were diagnosed within 1 year after blood draw also provided very similar results (eTable 2).

Table 1. Baseline and Clinical Characteristics of Study Participants

Discrete Variables	No. (%) of Participants in Group	
	Case (n = 899)	Control (n = 1815)
Participating countries		
Great Britain	176 (20)	356 (20)
Germany	161 (18)	319 (18)
Italy	139 (15)	278 (15)
Spain	130 (14)	259 (14)
The Netherlands	121 (13)	242 (13)
Greece	90 (10)	186 (10)
Sweden	58 (6)	127 (7)
France	24 (3)	48 (3)
Sex		
Men	559 (62)	1126 (62)
Women	340 (38)	689 (38)
Smoking status		
Never smokers	96 (11)	707 (39)
Former smokers	260 (29)	663 (37)
Years since quitting <10	132 (52)	179 (28)
Years since quitting ≥10	120 (48)	462 (72)
Current smokers	529 (59)	413 (23)
Unknown	14 (2)	32 (2)
Education		
Primary school	460 (53)	787 (45)
Technical/professional school	193 (22)	386 (22)
Secondary school	110 (13)	241 (14)
Higher education[a]	97 (11)	320 (18)
Body mass index[b]		
<20	40 (4)	42 (2)
≥20-<25	348 (39)	586 (32)
≥25-<30	383 (43)	873 (48)
≥30-<35	105 (12)	261 (14)
≥35	23 (3)	53 (3)
Alcohol intake at recruitment		
Never drinkers	34 (4)	88 (5)
Former drinkers	90 (10)	122 (7)
<5 g/d	268 (30)	574 (32)
≥5-<20 g/d	200 (22)	541 (30)
≥20 g/d	307 (34)	490 (27)
Continuous variables, median (5th-95th percentile)		
Age at blood draw, y	59 (43-73)	59 (43-73)
Serum levels for components of the 1-carbon metabolism		
Vitamin B_2, Riboflavin, nmol/L	17.1 (7.1-61.3)	19.7 (8.1-71.8)
Vitamin B_6, Pyridoxal 5′-phosphate, nmol/L	31.6 (13.2-87.9)	40.3 (16.9-116)
Folate, nmol/L	12.2 (5.3-32.5)	14.4 (6.6-38.8)
Vitamin B_{12}, cobalamin, pmol/L	350 (180-629)	343 (190-607)
Homocysteine, μmol/L	12.6 (8.1-23.3)	12.1 (8.0-20.4)
Methionine, μmol/L	27.4 (19.0-42.6)	29.2 (20.9-43.8)
Clinical characteristics, case participants only		
Age at diagnosis, median (range), y	64 (38-85)	
Months from blood draw to diagnosis, median (range)	62 (1-151)	
Distribution of months from blood draw to diagnosis, No. (%)		
1-35	232 (26)	
36-59	202 (22)	
60-83	223 (25)	
84-151	242 (27)	
Histology, No. (%)		
Small cell carcinoma	110 (12)	
Adenocarcinoma	272 (30)	
Large cell carcinoma	50 (6)	
Squamous cell carcinoma	200 (22)	
Other carcinoma	267 (30)	

SI conversion factors: To convert B_2 to μg/dL, divide by 26.6; B_6 to ng/mL, divide by 4.046; folate to ng/mL, divide by 2.266; B_{12} to pg/mL, divide by 0.7378; and methionine, divide by 67.02.
[a]Indicates completion. Higher education includes a university degree.
[b]Body mass index is calculated as weight in kilograms divided by height in meters squared.

After stratifying by smoking status, similar and consistent decreases in risk were observed for never smokers, former smokers, and current smokers for both B_6 and methionine, indicating that results were not due to a smoking-associated artifact (Table 2). For example, among never smokers, *P* for trend was .004 for B_6, and *P* for trend was .04 for methionine. A moderate lower risk was observed for increasing serum folate levels (fourth vs first quartile OR, 0.68; 95% CI, 0.51-0.90; *P* for trend = .001), although this association was restricted to former and current smokers, and was not apparent in never smokers (fourth vs first quartile OR, 0.84; 95% CI, 0.43-1.65; *P* for trend = .41). No significant trends in risk were observed overall for serum vitamin B_2 (*P* for trend = .11), B_{12} (*P* for trend = .06), or homocysteine (*P* for trend = .78). Regarding the additional measures of serum vitamin B_6 that were available, a similar lower risk was observed for pyridoxal (fourth vs first quartile OR, 0.51; 95% CI, 0.38-0.69; *P* for trend <.0009), although not for pyridoxic acid (fourth vs first quartile OR, 0.83; 95% CI, 0.60-1.14; *P* for trend = .30). Simultaneous adjustment for B_6 (as measured by pyridoxal 5′-phosphate), pyridoxal, and pyridoxic acid resulted in an unchanged estimate for B_6 (fourth vs first quartile OR, 0.43; 95% CI, 0.28-0.66) and no association for pyridoxal and pydidoxic acid.

We explored further the association of all B vitamins and metabolites after stratifying on various effect modifiers and estimating the OR for $\log_2$ of serum levels. This OR (OR_{log2}) may be interpreted as the relative risk associated with a doubling of the exposure level. OR_{log2} for B_6 overall was 0.74 (95% CI, 0.66-0.83; *P* for trend = 3×10^{-7}; eFigure 1). This result was consistent when stratified by potential effect modifiers including country, histology, smoking status, and time from blood draw to diagnosis. Similarly, the OR_{log2} for methionine overall was 0.51 (95% CI, 0.39-0.67; *P* for trend = 4×10^{-6}) and was not modified after stratification by potential effect modifiers (eFigure 2). Additional stratified analyses were conducted for B_2, folate, B_{12}, and homocysteine, and no apparent effect modification was observed (eFigure 3,

eFigure 4, eFigure 5, eFigure 6). The lower risk for folate (OR_{log2}=0.80; 95% CI, 0.71-0.90) was mainly restricted to former smokers (OR_{log2}=0.78; 95% CI, 0.64-0.95) and current smokers (OR_{log2}=0.76; 95% CI, 0.63-0.92).

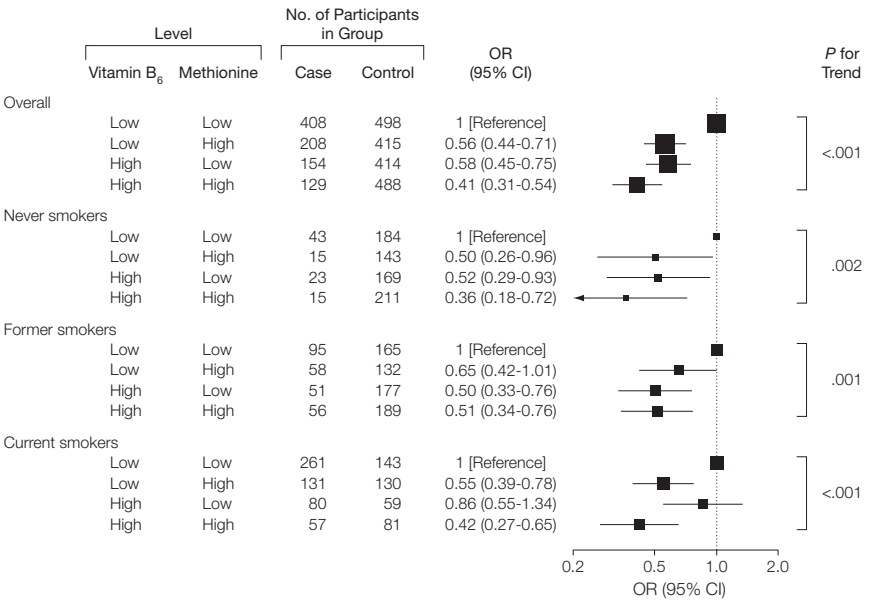
We further investigated the association of having a high level of B_6, methionine, or both by classifying individuals based on whether they were above or below the median values of these markers as measured among control participants (defined as <40.3nmol/L for B_6 and <29.2 µmol/L for methionine [FIGURE 1]). There were above-median values for both markers in 27% of control participants compared with only 14% of case participants (OR, 0.41; 95% CI, 0.31-0.54). Intermediate risks were obtained for participants who had low methionine but high B_6 levels (OR, 0.58; 95% CI, 0.45-0.75), as well as those who had high methionine and low B_6 levels (OR, 0.56; 95% CI, 0.44-0.71). The overall trend for having high levels of none, 1, or both measures was significant (*P* for trend = 3×10^{-12}). When stratifying by smoking, similar results for both high B_6 and methionine were observed among never (OR, 0.36; 95% CI, 0.18-0.72), former (OR, 0.51; 95% CI, 0.34-0.76), and current smokers (OR, 0.42; 95% CI, 0.27-0.65). When case and control participants were further classified according to the median level of folate in control participants (14.4 nmol/L), having above-median levels for all 3 vitamins resulted in an OR of 0.32 (95% CI, 0.23-0.45; eFigure 7).

Table 2. Odds Ratios of Lung Cancer for Serum Levels of Vitamins B_2, B_6, Folate, B_{12}, Homocysteine, and Methionine

		Odds Ratio (95% Confidence Interval)					
Quartile (Range)	Case/Control Participants[a]	Model 1[b] (n = 897/1768)[c]	Model 2[d] (n = 853/1621)[c]	Model 3[e] (n = 892/1748)[c]	Never Smokers[f] (n = 96/707)[g]	Former Smokers[f] (n = 260/663)[g]	Current Smokers[f,h] (n = 529/413)[g]
Vitamin B_2, riboflavin, nmol/L							
1 (2.9-13.3)	284/452	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]
2 (13.4-19.7)	237/451	0.92 (0.71-1.21)	0.90 (0.68-1.18)	1.03 (0.77-1.36)	1.06 (0.53-2.14)	0.87 (0.57-1.35)	0.77 (0.54-1.09)
3 (19.8-29.9)	185/450	0.83 (0.62-1.11)	0.85 (0.63-1.13)	0.98 (0.72-1.32)	1.14 (0.58-2.23)	0.80 (0.51-1.25)	0.80 (0.53-1.23)
4 (30.0-1433)	188/452	0.82 (0.61-1.09)	0.85 (0.63-1.14)	0.99 (0.73-1.35)	0.96 (0.48-1.91)	0.94 (0.60-1.46)	0.91 (0.58-1.43)
P for trend[i]		.11	.22	.81	.38	.66	.46
Vitamin B_6, pyridoxal 5′-phosphate, nmol/L							
1 (5.7-28.4)	380/452	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]
2 (28.5-40.3)	231/451	0.78 (0.60-1.01)	0.76 (0.57-1.00)	0.83 (0.63-1.10)	0.76 (0.41-1.40)	0.78 (0.52-1.18)	0.68 (0.47-0.97)
3 (40.4-57.7)	152/450	0.53 (0.40-0.71)	0.54 (0.40-0.73)	0.59 (0.43-0.80)	0.57 (0.30-1.09)	0.51 (0.33-0.80)	0.64 (0.42-0.98)
4 (57.8-629)	131/452	0.44 (0.33-0.60)	0.43 (0.32-0.59)	0.50 (0.36-0.69)	0.37 (0.17-0.77)	0.50 (0.32-0.77)	0.67 (0.42-1.06)
P for trend[i]		2×10^{-7}	5×10^{-7}	2×10^{-5}	.004	.006	.04
Folate, nmol/L							
1 (0.1-10)	326/452	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]
2 (10.1-14.4)	221/455	0.84 (0.64-1.10)	0.87 (0.66-1.15)	0.85 (0.64-1.12)	1.23 (0.64-2.36)	0.68 (0.44-1.05)	0.92 (0.64-1.32)
3 (14.5-22.4)	193/452	0.78 (0.59-1.03)	0.80 (0.60-1.07)	0.81 (0.60-1.10)	0.90 (0.47-1.75)	0.75 (0.49-1.15)	0.84 (0.56-1.26)
4 (22.5-395)	159/455	0.68 (0.51-0.90)	0.68 (0.50-0.91)	0.69 (0.50-0.95)	0.84 (0.43-1.65)	0.58 (0.37-0.91)	0.54 (0.34-0.83)
P for trend[i]		.001	.002	.008	.41	.02	.003
Vitamin B_{12}, cobalamin, pmol/L							
1 (28.7-274)	227/453	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]
2 (275-343)	199/454	0.94 (0.72-1.25)	0.97 (0.73-1.29)	0.95 (0.71-1.26)	1.00 (0.50-1.99)	0.86 (0.57-1.31)	0.92 (0.61-1.38)
3 (344-427)	240/454	1.29 (0.99-1.70)	1.33 (1.01-1.76)	1.32 (0.99-1.75)	0.78 (0.40-1.51)	1.26 (0.83-1.90)	1.49 (1.00-2.24)
4 (428-3800)	233/454	1.22 (0.92-1.62)	1.20 (0.90-1.59)	1.35 (1.00-1.82)	0.80 (0.41-1.54)	1.39 (0.92-2.11)	1.29 (0.84-1.98)
P for trend[i]		.06	.08	.04	.37	.05	.05
Homocysteine, µmol/L							
1 (4.9-10.3)	202/450	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]
2 (10.4-12.1)	197/452	0.83 (0.62-1.12)	0.79 (0.59-1.06)	0.81 (0.60-1.10)	0.73 (0.41-1.31)	0.80 (0.50-1.27)	1.11 (0.71-1.74)
3 (12.2-14.5)	227/451	0.85 (0.63-1.15)	0.84 (0.62-1.15)	0.73 (0.53-1.01)	0.62 (0.32-1.19)	0.78 (0.49-1.24)	1.00 (0.64-1.57)
4 (14.6-139)	270/452	0.87 (0.64-1.17)	0.82 (0.60-1.12)	0.76 (0.54-1.07)	0.42 (0.19-0.90)	0.92 (0.58-1.46)	1.05 (0.67-1.66)
P for trend[i]		.78	.84	.62	.03	.71	.38
Methionine, µmol/L							
1 (11-25.3)	309/451	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]
2 (25.4-29.1)	250/451	0.88 (0.68-1.15)	0.90 (0.69-1.18)	0.91 (0.69-1.18)	1.30 (0.73-2.30)	0.69 (0.46-1.03)	1.25 (0.84-1.87)
3 (29.2-33.9)	176/451	0.49 (0.36-0.65)	0.51 (0.38-0.69)	0.51 (0.38-0.69)	0.79 (0.41-1.52)	0.71 (0.46-1.10)	0.55 (0.36-0.81)
4 (34.0-69.2)	161/452	0.52 (0.39-0.69)	0.53 (0.40-0.72)	0.54 (0.40-0.73)	0.48 (0.22-1.04)	0.57 (0.37-0.88)	0.66 (0.43-1.01)
P for trend[i]		3×10^{-7}	2×10^{-6}	5×10^{-6}	.04	.005	.02

SI conversion factors: To convert B_2 to µg/dL, divide by 26.6; B_6 to ng/mL, divide by 4.046; folate to ng/mL, divide by 2.266; B_{12} to pg/mL, divide by 0.7378; and methionine, divide by 67.02.
[a] Numbers include all case and control participants for whom laboratory measurements were available, including those participants from uninformative case sets excluded from conditional analyses.
[b] Assessed by conditional logistic regression conditioning on individual case set adjusting for cotinine (in quartiles).
[c] Numbers (n=case/control participants) only include participants from informative case sets, ie, those from case sets with at least 1 case and 1 control participant.
[d] Further adjusted for body mass index (in quartiles), educational attainment (in 5 groups), and alcohol intake at recruitment (in quartiles).
[e] Adjusted for cotinine (in quartiles) and mutually adjusted for all the other analytes simultaneously (in quartiles).
[f] Assessed by unconditional logistic regression adjusting for age (in 5-year categories), country, and sex.
[g] Numbers (n=case/control participants) also include participants from uninformative case sets who were excluded from conditional analyses.
[h] Further adjusted for cotinine (in quartiles).
[i] *P* for trend assessed by the base 2 logarithm of the serum levels.

Figure 1. Odds Ratios of Lung Cancer for Groups of Serum Vitamin B_6 and Methionine Adjusted for Cotinine



High/low for vitamin B_6 denotes levels above/below 29.2 nmol/L, high/low for methionine denotes serum levels above/below 40.3 μmol/L. Odds ratios (ORs) were assessed by unconditional logistic regression adjusting for age (in 5-year categories), country, sex, and cotinine (in quartiles); *P* for trend was assessed by a discrete variable (0-2) indicating if 1 or 2 of the analytes are high; the black squares and horizontal lines indicate the ORs and 95% confidence intervals (CIs); the size of the black squares is proportional to the inverse variance of the logistic regression estimates.

Dietary Intake of B Vitamins and Lung Cancer Risk

Smokers consumed fewer fruits and vegetables than did never and former smokers (eTable 3). There were weak correlations between dietary vitamin measures and serum levels for B_2, B_6, and B_{12} (eTable 4), consistent with observations in other studies.[22-24] In the comparison between serum B_6 and dietary B_6 as assessed from the food frequency questionnaires among the same group of case and control participants, while a decreasing risk was observed with increasing levels of serum B_6, no association was observed with dietary B_6 either overall (*P* for trend = .81), or among the second (OR, 0.91), third (OR, 0.99), and fourth quartile (OR, 1.02; eTable 5). A potential increased risk was observed with increasing levels of dietary B_{12} (*P* for trend = .007) that mirrored the nonsignificant increase observed for serum B_{12} levels (*P* for trend = .06). No association was observed for dietary intake of B_2, similar to the results for serum B_2 concentrations.

Cumulative Risks of Lung Cancer

Using the sex, smoking, and age-specific incidence rates within EPIC, we obtained cumulative risks of lung cancer (by age 79 years in the absence of other causes of death) among never, former, and current smokers of 0.50%, 3.4%, and 10.8% for men, and 0.47%, 1.6%, and 6.9% for women, respectively. Cumulative risks were subsequently calculated separately for participants with above-median and below-median serum levels of both B_6 and methionine (FIGURE 2). Among current smokers, cumulative risks ranged from 14.9% for men with above-median levels of both and 6.6% for men with below-median levels of both. The corresponding estimates for women were 8.9% and 3.8%, respectively. Cumulative risks among former-smoking men were 5.2% and 2.7%, respectively, and 2.5% and 1.3% among women, respectively. Among never-smoking men, cumulative risks were 0.90% and 0.32%, respectively, and 0.75% and 0.27% among women, respectively. Similar results were obtained for quartiles of B_6, folate, and methionine separately (eTable 6).

COMMENT

Our results suggest that above-median serum measures of both B_6 and methionine, assessed on average 5 years prior to disease onset, are associated with a reduction of at least 50% on the risk of developing lung cancer. An additional association for serum levels of folate was present, that when combined with B_6 and methionine, was associated with a two-thirds lower risk of lung cancer.

Reverse Causation and Confounding by Smoking

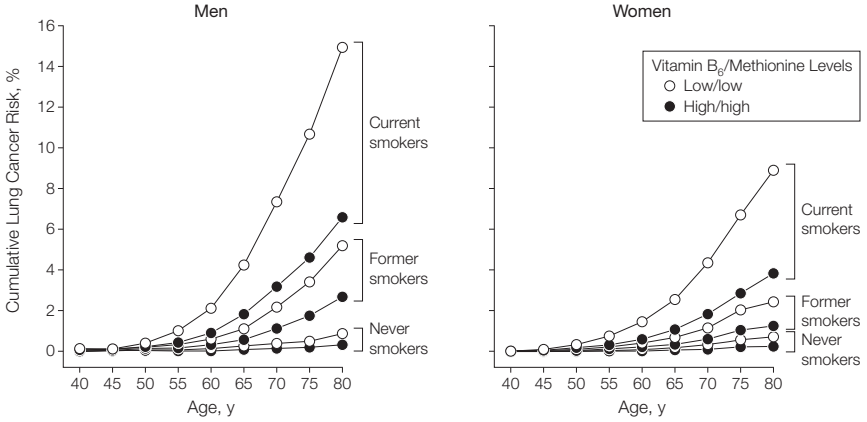
A noncausal explanation for the observed results is that of reverse causation, ie, underlying preclinical disease is suppressing serum levels of both B_6 and methionine. If this were the case, one would expect greater associations in the initial periods after blood collection, when preclinical disease might be most apparent. The OR for both serum B_6 and methionine were, however, very stable over the 12-year follow-up after blood collection, which would seem to exclude any possible reverse causation bias (eFigure 1; eFigure 2). As a further check against any possible reverse causation, we repeated analysis after excluding participants who developed lung cancer within 1 year of blood collection. Overall results for B_6 (*P* for trend $<.000001$) and methionine (*P* for trend = .00001) were almost identical to those including all cases (eTable 2).

A second possible noncausal explanation is that the results are confounded by cigarette smoking. Again, there are a number of reasons why this does not appear to be a plausible explanation of the results. First, when we compared various lifestyle exposures with serum levels among all participants, current smoking appeared to be associated with all serum measures, including those that were not subsequently associated with lung cancer (eTable 1). The strongest associated measures among current smokers were serum vitamin B_6 (concentration ratio [CR] compared with never smokers, 0.78; $P<10^{-15}$), B_2 (CR, 0.79; $P<10^{-11}$), homocysteine (CR, 1.09; $P=10^{-9}$), and folate

(CR, 0.86; $P<10^{-6}$). Serum vitamin B_{12} measures were moderately suppressed (CR, 0.94; $P=.001$), although the least-affected measure was for methionine (CR, 0.97; $P=.01$). Second, former smoking did not appear to suppress any of the 6 serum measures, with average levels being very similar between former and never smokers (eg, CR=0.99 and 1.00 for B_6 and methionine, respectively). From these data, one might conclude that serum measures among former smokers are not confounded by smoking status, although serum measures among current smokers are. Similarly, when comparing dietary intake of major food groups across smoking categories, no differences between never and former smokers were observed, whereas current smokers consumed lower levels of both fruits and vegetables (eTable 3).

Third, an association among never smokers, as is apparent for both B_6 and methionine, would appear to rule out confounding by smoking as an explanation for the association with both. Smoking cannot therefore explain the association with never smokers, and is unlikely to explain the association among former smokers. Among current smokers, some confounding is plausible, although given that we have been able to adjust for cotinine, the most parsimonious explanation would seem to be that the results among never, former, and current smokers are roughly equivalent.

Figure 2. Cumulative Risks of Lung Cancer to Age 79 Years Among Never, Former, and Current Smokers



Cumulative risks up to age 79 were based on age-specific incidence rates estimated within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort, separately for men and women, and among never, former, and current smokers, combined with odds ratio estimates of lung cancer by median serum levels of B_6 and methionine. High/high indicates having above-median levels of both vitamin B_6 and methionine and low/low indicates having below-median levels.

Other Potential Confounders

An additional noncausal explanation is that the observed associations are confounded by other risk factors for lung cancer, including occupation or markers of social deprivation. Serum methionine appeared to be unrelated to most potential confounders for which information in the EPIC cohort was available including employment status, physical activity, BMI, and alcohol consumption (eTable 1). There was a small increase in serum methionine with higher educational attainment (CR, 1.01; $P=.002$), although any further adjustment for this and BMI had no material effect on the overall OR (Table 2). Similarly, although baseline levels of serum vitamin B_6 were associated with educational attainment, physical activity, and with alcohol consumption, adjustment for these variables had no effect on the observed OR (Table 2). Given the absence of any apparent confounding effect from these exposures, residual confounding from poorly measured exposures would also appear to be unlikely. This leaves the possibility that other unidentified exposures for lung cancer (eg, specific occupational exposures), are strongly associated with both serum B_6 and methionine levels, and explain the observed results. Given their joint correlation was also limited ($\rho=.17$; eTable 7), any unknown confounder of these associations would have to be strongly associated with both, as well as with lung cancer. We would therefore argue that, having excluded smoking, other potential confounders that explain these associations are unlikely.

Independent and Combined Associations of Serum B_6, Methionine, and Folate

Serum markers of B vitamins and related metabolites have been assessed for multiple cancer sites in prospective cohorts, including colorectal,[25-30] gastric,[31] pancreatic,[32] prostate,[33,34] and breast cancer.[35-37] Studies of colorectal cancer and serum vitamin B_6 would seem to provide very consistent evidence of a protective association in the order of 50% lower risk when comparing the fourth quartile with the first quartile of the exposure distribution. Our results showing a lower risk of lung cancer with increasing serum vitamin B_6 status are therefore consistent with observed results from other large cohort studies for colorectal cancer, although evidence is limited for other cancer types. Previous studies on colorectal and other cancers have not investigated a potential role for serum methionine levels, and to our knowledge no previous studies have reported on the combined association of serum vitamin B_6, folate, and methionine.

These epidemiological findings, as well as results from animal studies,[38] have led to the conduct of 2 large randomized studies aiming to test whether folate supplementation reduces the risk of colorectal cancer.[11,12] Neither study provided any positive evidence of a reduced risk of colorectal adenomas among participants randomized to receive folic acid supplementation, with some evidence in the US study of an increased risk of advanced or multiple adenomas. These results have led to the hypothesis that timing of folate supplementation may be essential, with folate being beneficial in primary prevention of

colorectal neoplasia, but potentially harmful in the presence of established cancer.[39] Similarly, it is also plausible that any effect with B_6 and methionine may be modified by stage of disease.

Regression Dilution

Our serum B vitamin measurements were performed on a single blood sample obtained at study recruitment and as such are likely to be imperfect estimates of underlying historical exposure level. The correlation between our estimate and the underlying long-term level is likely to depend on a number of factors including day-to-day, seasonal, and more long-term variation within an individual. The consequence of this is that our estimated OR will be weaker than the true underlying association. It is possible, if one has multiple blood samples taken preferably many years apart, to correct for this "regression dilution."[40] As an indication of the extent to which our OR estimates might be attenuated by regression dilution, we acquired repeat measurements taken 1 year apart for serum vitamin B_6, methionine, and folate from the control group (n=755) of the Western Norway B-vitamin Intervention Trial (WENBIT).[41] This resulted in corrected OR estimates for all 3 measures that were substantially lower than uncorrected measures, although particularly so for methionine (eTable 8). For example, comparing the fourth and first quartile of vitamin B_6, the corrected OR was 0.25 for vitamin B_6, 0.47 for folate, and 0.13 for methionine. Similar results were obtained for repeat measures taken 1 month and 3 years apart (eTable 8). Given that the combined OR of having above-median levels of all 3 measures was 0.32 (eFigure 7), these results indicate that the true underlying association is likely to be much stronger.

Comparison of Food Frequency Questionnaires and Serum Measures

We observed no association between vitamin B_6 estimated from the food frequency questionnaires and lung cancer risk, in contrast to the strong protective association observed from serum levels (eTable 5). There are at least 2 possible interpretations for this discrepancy. One is that serum measures are a far more accurate reflection of vitamin B_6 intake than estimates based on multiple food types determined by questionnaire. The correlation of 0.16 between the food frequency questionnaires and serum levels, similar to that observed in other studies,[22-24] would be in line with this. An alternative explanation is that serum levels of vitamin B_6 differ strongly between case and control participants not because of intake but because of absorption, distribution, or catabolism of the circulating nutrient. This will result in lower serum levels among the case participants even when intake is similar. An additional consequence would be that dietary modification would not be a suitable means for reducing cancer incidence. Assuming the associations with B_6 are causal, identifying which of these 2 explanations is true will be crucial.

Public Health

Dietary sources of B_6 are varied and include beans, grains, meats, poultry, fish, and some fruits and vegetables, whereas primary sources of methionine are from animal proteins, as well as some nuts and vegetable seeds.[3] Given that serum levels of B vitamins and metabolites are at least partially determined by diet (eTable 4), and are clearly affected by vitamin supplements,[41] low vitamin levels are therefore modifiable. However, based on the recent experience of folate intervention trials for colorectal adenomas, as well as past intervention trials for lung cancer,[11,12] it is unlikely that further intervention trials of B vitamins would be advisable. A recent pooled analysis of 2 randomized trials reported a potential excess in risk for all cancers combined and lung cancer among participants randomized to receive folic acid and B_{12}, with no apparent effect for B_6.[42] These results would further support the hypothesis that randomization to B vitamins over several years does not provide any short-term benefits in cancer reduction, although do not inform about potential protective effects regarding maintaining adequate serum levels of B vitamins over the life course.[43]

If our observations regarding serum methionine, B_6, or both are shown to be causal, identifying optimum levels for reducing future cancer risk would appear to be appropriate. It is also possible that one may be able to obtain further evidence of potential causal effects, at least for B_6, by analyzing modifier genes that have recently been identified from genome-wide studies of vitamin B serum levels.[8] Given the modest effect on serum levels associated with these gene variants, very large sample sizes will be required in order to obtain robust results.

Lung cancer remains the most common cause of cancer death in the world today and is likely to remain so for the near future.[44] It is essential that for lung cancer prevention, any additional evidence about causality does not detract from the importance of reducing the numbers of individuals who smoke tobacco. With this in mind, it is important to recognize that a large proportion of lung cancer cases occur among former smokers, making up the majority in countries where tobacco campaigns have been particularly successful, and a nontrivial number of lung cancer cases occur also among never smokers, particularly among women in parts of Asia.[45-47] Clarifying the role of B vitamins and related metabolites in lung cancer risk is likely therefore to be particularly relevant for former smokers and never smokers.

Author Contributions: Dr Brennan had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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Critical revision of the manuscript for important intellectual content: Johansson, Relton, Ueland, Vollset, Midttun, Nygård, Slimani, Boffetta, Jenab, Clavel-Chapelon, Boutron-Ruault, Fagherazzi, Kaaks, Rohrmann, Boeing, Weikert, Bueno-de-Mesquita, Ros, van Gils, Peeters, Agudo, Barricarte, Navarro, Rodríguez, Sánchez, Larrañaga, Khaw, Wareham, Allen, Crowe, Gallo, Norat, Krogh, Masala, Panico, Sacerdote, Tumino, Trichopoulou, Lagiou, Trichopoulos, Rasmuson, Hallmans, Riboli, Vineis, Brennan.
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Obtained funding: Nygård, Slimani, Boffetta, Jenab, Clavel-Chapelon, Boutron-Ruault, Fagherazzi, Kaaks, Rohrmann, Boeing, Weikert, Bueno-de-Mesquita, Ros, van Gils, Peeters, Agudo, Barricarte, Navarro, Rodríguez, Sánchez,

Larrañaga, Khaw, Wareham, Allen, Crowe, Gallo, Norat, Krogh, Masala, Panico, Sacerdote, Tumino, Trichopoulou, Lagiou, Trichopoulos, Rasmuson, Hallmans, Riboli, Vineis, Brennan.

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Study supervision: Johansson, Relton, Ueland, Vollset, Midttun, Vineis, Brennan.

Drs Johansson and Relton both contributed equally to this article.

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Online-Only Materials: eTables 1-8 and eFigures 1-7 are available at http://www.jama.com.

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