DOI:10.1093/jnci/dju327
First published online November 5, 2014

ARTICLE

Circulating Biomarkers of One-Carbon Metabolism in Relation to Renal Cell Carcinoma Incidence and Survival

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Manuscript received December 31, 2013; revised April 11, 2014; accepted September 3, 2014.

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Background The etiology of renal cell carcinoma (RCC) is only partially understood, but a metabolic component appears likely. We investigated biomarkers of one-carbon metabolism and RCC onset and survival.

Methods The European Prospective Investigation into Cancer and Nutrition (EPIC) recruited 385 747 participants with blood samples between 1992 and 2000, and this analysis included 556 RCC case-control pairs. A subsequent replication study included 144 case-control pairs nested within the Melbourne Collaborative Cohort Study (MCCS). Plasma concentrations of vitamin B2, vitamin B6, folate, vitamin B12, methionine and homocysteine were measured in prediagnostic samples and evaluated with respect to RCC risk using conditional and unconditional logistic regression models, and to all-cause mortality in RCC cases using Cox regression models. All statistical tests were two-sided.

Results EPIC participants with higher plasma concentrations of vitamin B6 had lower risk of RCC, the odds ratio comparing the 4th and 1st quartiles (OR_{4vs1}) being 0.40 95% confidence interval [CI] = 0.28 to 0.57, $P_{trend} < .001$. We found similar results after adjusting for potential confounders (adjusted $P_{trend} < .001$). In survival analysis, the hazard ratio for all-cause mortality in RCC cases when comparing the 4th and 1st quartiles (HR_{4vs1}) of vitamin B6 was 0.57 (95% CI = 0.37 to 0.87, $P_{trend} < .001$).

Subsequent replication of these associations within the MCCS yielded very similar results for both RCC risk (OR_{4vs1} = 0.47, 95% CI = 0.23 to 0.99, P_{trend} = .07) and all-cause mortality (HR_{4vs1} = 0.56, 95% CI = 0.27 to 1.17, P_{trend} = .02). No association was evident for the other measured biomarkers.

Conclusion Study participants with higher circulating concentrations of vitamin B6 had lower risk of RCC and improved survival following diagnosis in two independent cohorts.

JNCI J Natl Cancer Inst (2014) 106(12): dju327 doi:10.1093/jnci/dju327

The etiology of kidney cancer is not well understood, and there are notable unexplained differences in incidence. The highest rates worldwide are observed in the Czech Republic (1), and in the United States; the age-standardized rates (ASR) of kidney cancer are approximately two-fold higher for African Americans and European Americans than for Asians (2). Renal cell carcinoma (RCC) is the predominant type of kidney cancer and accounts for approximately 80% of cases (2).

Established risk factors for RCC include tobacco smoking, obesity, and hypertension, as well as recently discovered gene variants (2–4). Additionally, it has been suggested that diabetes mellitus may increase RCC risk, whereas lifestyle factors such as high physical activity, alcohol, and intake of fruits and vegetables may reduce risk (2). The relation between fruit and vegetable intake and RCC is intriguing and consistently observed in both retrospective and prospective case-control studies (5). While residual confounding

by tobacco smoking is of concern in the interpretation of these studies, a causal association cannot be excluded (6–8).

Fruits and vegetables are sources of B vitamins and other components of the one-carbon metabolism pathway, which is important in maintaining DNA methylation and DNA repair mechanisms in the body (9,10). Circulating concentrations of B-vitamins have been investigated in relation to multiple cancers, but only one prospective study has been published for RCC (11).

We sought to investigate whether concentrations of circulating B-vitamins and amino acids in the one-carbon metabolism pathway are related to RCC incidence and outcome using a large European cohort. To ensure the validity of our findings, we also conducted a replication of promising results within a separate Australian cohort.

Methods

Study Cohort—The European Prospective Investigation into Cancer and Nutrition (EPIC)

The EPIC study recruitment procedures have been previously described in detail (12), and important cohort information and follow-up procedures are provided in the Supplementary Methods (available online).

Selection of Cases and Controls

We initially identified 905 cases within EPIC that were diagnosed with RCC as C64.9 according to the International Classification of Diseases for Oncology, Second Edition (ICD-O-2). After excluding prevalent cases and cases with a prior history of another cancer (n = 85, except nonmelanoma skin cancer), cases who did not donate a blood sample (n = 153), were not histologically confirmed (n = 27), did not have questionnaire information available (n = 6), and cases from the Malmö center that did not participate in this study (n = 64), 570 RCC cases remained eligible.

For each case, one control was randomly chosen from risk sets consisting of all cohort members alive and free of cancer (except nonmelanoma skin cancer) at the time of diagnosis of the index case. Matching criteria were: country, sex, date of blood collection (± one month, relaxed to ± five months for sets without available controls), and date of birth (± one year, relaxed to ± five years). Additionally, we included 553 controls (control group 2) matched to cases of a parallel study of head and neck cancer using identical matching criteria. Biochemical analyses were undertaken in the same laboratory under the same conditions, and at the same time for all cases and controls.

After excluding sets with only one case or control, 556 case-control sets remained, as well as 553 additional unmatched controls from control group 2 that contributed to unconditional and stratified analyses.

Replication Study—The Melbourne Collaborative Cohort Study (MCCS)

To replicate promising associations, we designed a case-control study nested within the MCCS. Extensive details on recruitment and follow-up have been published previously (also see Supplementary Methods, available online) (13,14). Incident cases and controls were selected using the same protocol as the EPIC study. A total of 144 case-control pairs were available. The MCCS data were used as an independent replication analysis and were not pooled with the EPIC data.

Biochemical Analyses

Plasma samples were sent on dry ice to the Bevital A/S laboratory (http://www.bevital.no) in Bergen, Norway, where vitamin B2 (riboflavin), vitamin B6 (measured as pyridoxal 5'-phosphate, its active form), folate (vitamin B9), vitamin B12 (cobalamin), total homocysteine, and methionine were measured. Cotinine was measured as an indicator of recent smoking behavior. Details of the biochemical analyses are provided in Supplementary Methods (available online) (15–19).

Statistical Analyses

Quartiles of plasma concentrations for each biomarker were calculated based on the distribution among controls. Odds ratios (ORs) and 95% confidence intervals (CIs) of RCC were calculated relative to the first quartile using conditional logistic regression, conditioning on individual case set. Log-linear trends (P_{trend}) were calculated by including the base 2 logarithm ($\log_2$) of the biomarker concentration as a continuous variable in separate models.

To assess the consistency of any association, we compared RCC cases with the additional unmatched controls (control group 2) using unconditional logistic regression, adjusting for sex, country, and age at recruitment (five-year groups). We also compared the RCC cases with all EPIC controls in order to increase the statistical power.

To evaluate whether known risk factors of RCC could explain any association, we assessed if OR estimates were affected after including indicator variables in the logistic regression models for tobacco smoking (smoking status at baseline [never, former, current] and quartiles of cotinine concentrations [determined by the distribution for current smokers]), alcohol intake at recruitment (g/day), lifetime alcohol intake [ever/never], obesity (indicated by body mass index [BMI], five categories: <18.5, 18.5–25, 25–30, 30–35, ≥35 kg/m^2), educational attainment (in four categories), waist-to-hip ratio (quartiles), and self-reported history of hypertension (yes/no). Missing values for these covariates were imputed under a multivariable normal model. One hundred imputations were drawn from the imputation model, which included all covariates and biomarkers, as well as the case-control status. For binary and categorical covariates, we rounded imputed values to the nearest integer category (20,21). As a sensitivity analysis, we also fitted models excluding observations with missing covariate data.

To assess potential effect modification, we performed stratified analyses by country of recruitment, smoking status, age at diagnosis, sex, education, time from blood draw to diagnosis, and waist-to-hip ratio. These analyses included the $\log_2$ of each biomarker as a continuous covariates in unconditional logistic regression models. χ^2 tests were applied to assess heterogeneity between ORs.

Hazard ratios (HRs) for all-cause mortality for RCC cases were calculated using Cox proportional hazards models. Time since diagnosis was used as the timescale, and all models were adjusted for age at diagnosis, sex, and country. Further adjustment was undertaken for tobacco smoking, alcohol intake at recruitment,

education, hypertension, and waist-to-hip ratio. Tests for trend were based on models including $\log_2$ of the biomarker concentrations. Visual inspection of smoothed, scaled Schoenfeld residuals revealed no notable departure from proportional hazards. Model based estimates of the survival function by biomarker quartiles were calculated using flexible parametric survival models (22). Restricted cubic splines with five knots (placed at evenly spaced centiles of the uncensored log survival times) were used to model the baseline hazard.

Among the matched controls, partial correlation coefficients (conditional on country, age, and sex) were calculated between log-transformed biomarker concentrations and dietary intake of fruits, vegetables, meats, and dairy products, as well as alcohol intake, circulating cotinine, BMI, and waist-to-hip ratio. All *P* values were two-sided. Statistical analyses were conducted using SAS 9.2 (Cary, NC) and Stata 12.1 for Linux (Stata Corporation).

Results

The EPIC Study Population

Baseline and demographic characteristics of the EPIC study population are displayed in Table 1. Of the individually matched cases and controls, 56% were men and 44% were women. Median age at recruitment was 57 years (5^{th}-95^{th} percentile: 42–67), and average time from blood draw to RCC diagnosis was 6.7 years. Control group 2 included an additional 553 subjects with similar demographic characteristics as the matched control group, albeit with a higher proportion of men (68%). Known risk factors of RCC displayed expected differences between cases and controls, including waist-to-hip ratio, BMI, smoking status, and hypertension. No substantial correlations were observed between one-carbon metabolism biomarkers and dietary intake of major food groups, circulating cotinine, alcohol intake, BMI, or waist-to-hip ratio (Supplementary Table 1, available online).

Plasma Concentrations of One-Carbon Metabolism Biomarkers and Risk of RCC

Initial conditional risk analysis was conducted by comparing cases and matched controls for quartiles of plasma biomarkers of one-carbon metabolism (Table 2). Concentrations of vitamin B2, folate, B12, homocysteine and methionine displayed weak or no evidence of association with RCC risk ($P_{trend} > .06$). In contrast, participants with higher concentrations of vitamin B6 had a lower risk of RCC in a dose response fashion ($P_{trend} < .001$), the OR when comparing the 4^{th} and 1^{st} quartiles (OR_{4vs1}) being 0.40 (95% CI = 0.28 to 0.57). Adjusting for potential confounders did not notably affect the OR estimates (adjusted OR_{4vs1} = 0.43, 95% CI = 0.29 to 0.64, $P_{trend} < .001$) (Table 2). In a sensitivity analysis in which participants with missing covariate information were excluded, the corresponding adjusted OR_{4vs1} was 0.34 (95% CI = 0.21 to 0.57, $P_{trend} < .001$) (Supplementary Table 2, available online).

After accounting for vitamin B6, the other plasma biomarkers (vitamin B2, folate, B12, homocysteine and methionine) did not display any association with RCC risk (data not shown).

When comparing RCC cases with control group 2, we observed similar associations (Supplementary Table 2, available online). After combining all EPIC controls in an unconditional analysis the unadjusted OR_{4vs1} was 0.43 (95% CI = 0.32 to 0.60, $P_{trend} < .001$) and 0.49 (95% CI = 0.35 to 0.68, $P_{trend} < .001$) after adjusting for risk factors (Supplementary Table 2, available online).

Replication Study

In order to determine if the inverse relation between vitamin B6 and risk of RCC was restricted to the EPIC study population, we analyzed plasma concentrations of vitamin B6 and cotinine in 144 additional case-control pairs nested within the MCCS. The distribution of vitamin B6 within the MCCS was similar to that within EPIC; hence, the same quartile cutoff points were applied. We obtained very similar OR estimates of RCC for quartiles of vitamin B6 within the MCCS, the OR_{4vs1} being 0.47 (95% CI = 0.23 to 0.99), after adjusting for available risk factors (Table 3).

Associations With a Doubling of Plasma Vitamin B6 Levels

A doubling in plasma vitamin B6 was associated with a 20% lower odds of RCC in MCCS (OR for $\log_2$B6 [OR_{log2}] = 0.80, 95% CI = 0.64 to 1.02, P_{trend} = .07) and a 22% lower odds in EPIC (OR_{log2} = 0.78, 95% CI = 0.63 to 0.82, $P_{trend} < .001$). The corresponding unadjusted OR_{log2} estimates after stratifying by various descriptive variables within EPIC are displayed in Figure 1. The association between concentrations of vitamin B6 and RCC risk was marginally more prominent for men than women, for current smokers than former and never smokers, and for subjects that were not hypertensive. The OR_{log2} for vitamin B6 among current smokers, after adjusting for hypertension, waist-to-hip ratio, educational attainment, alcohol intake, BMI, and circulating cotinine was 0.52 (95% CI = 0.39 to 0.71). Additional adjustment for number of cigarettes smoked per day and duration of smoking did not affect the estimate (OR_{log2} = 0.53, 95% CI = 0.39 to 0.72). We further note that the association between vitamin B6 and risk was evident when evaluating blood samples taken up to ten years prior to diagnosis (Figure 1). Circulating concentrations of vitamin B6 by demographic variables, risk factors, and tumor stage are provided in Supplementary Table 3 (available online).

All-Cause Mortality for RCC Cases

Results of Cox proportional hazards regression for all-cause mortality (205 deaths in total) are shown in Table 4 and Supplementary Table 4 (available online). For vitamin B6 the HR for all-cause mortality for RCC cases when comparing the 4th and 1st quartiles was 0.57 (95% CI = 0.37 to 0.87) (Table 4). The corresponding P_{trend} was less than .001, and the trend HR for $\log_2$B6 (HR_{log2}) was 0.74 (95% CI = 0.62 to 0.88). This result was nearly identical when excluding cases diagnosed within two years of blood draw (HR_{log2} = 0.73, 95% CI = 0.60 to 0.90, P_{trend} = .003). For RCC cause-specific mortality (147 deaths), the HR_{4vs1} was 0.36 (95% CI = 0.20 to 0.65), the corresponding HR_{log2} was 0.64 (95% CI = 0.52 to 0.79, P_{trend} <.001). For comparison, we observed 36 deaths among the matched controls and the corresponding HR_{log2} of all-cause mortality was 1.01 (95% CI = 0.71 to 1.45, P_{trend} = .95). Adjusting the all-cause mortality

Table 1. Baseline and demographic characteristics of the study participants from EPIC

	RCC cases	Matched controls	Control group 2
Continuous variables	**No. (%), n = 556**	**No. (%), n = 556**	**No. (%), n = 553**
Sex*			
Men	310 (56%)	310 (56%)	374 (67.6%)
Women	246 (44%)	246 (44%)	179 (32.4%)
Participating countries*			
France	13 (2%)	13 (2%)	7 (1.3%)
Italy	88 (16%)	88 (16%)	70 (13%)
Spain	52 (9%)	52 (9%)	100 (18%)
United Kingdom	67 (12%)	67 (12%)	130 (24%)
The Netherlands	46 (8%)	46 (8%)	77 (14%)
Greece	17 (3%)	17 (3%)	22 (4%)
Germany	125 (22%)	125 (22%)	104 (19%)
Sweden	32 (6%)	32 (6%)	41 (7%)
Denmark	112 (20%)	112 (20%)	
Norway	4 (1%)	4 (1%)	2 (0.4%)
Smoking status			
Never smokers	225 (41%)	244 (44%)	230 (42%)
Former smokers	160 (29%)	180 (32%)	199 (36%)
Years since quitting ≥10	94 (58%)	122 (68%)	135 (68%)
Years since quitting <10	66 (42%)	58 (32%)	64 (33%)
Current smokers	166 (30%)	129 (23%)	110 (20%)
Unknown	5 (1%)	3 (1%)	14 (3%)
Educational attainment			
Primary school	229 (41%)	206 (37%)	222 (40%)
Technical/professional school	124 (22%)	136 (25%)	141 (26%)
Secondary school	77 (14%)	66 (12%)	70 (13%)
Higher education	110 (20%)	134 (24%)	99 (18%)
Unknown	16 (3%)	14 (3%)	21 (4%)
Body mass index, kg/m^2			
<18.5	2 (0%)	2 (0%)	5 (1%)
18.5–25	179 (32%)	221 (40%)	215 (39%)
25–30	247 (45%)	241 (43%)	258 (47%)
30–35	99 (18%)	69 (12%)	61 (11%)
≥35	29 (5%)	23 (4%)	14 (3%)
Waist-to-hip ratio			
0.56–0.79	92 (17%)	113 (20%)	86 (16%)
0.80–0.89	129 (23%)	145 (26%)	146 (26%)
0.90–0.94	103 (19%)	114 (21%)	128 (23%)
0.95–1.30	196 (35%)	148 (27%)	150 (27%)
Unknown	36 (7%)	36 (7%)	43 (8%)
Hypertension			
No	276 (50%)	325 (59%)	318 (58%)
Yes	192 (35%)	140 (25%)	115 (21%)
Unknown	88 (16%)	91 (16%)	120 (22%)
Alcohol intake			
Never drinkers	37 (7%)	22 (4%)	28 (5%)
Ever drinkers	509 (92%)	530 (95%)	520 (94%)
Unknown	10 (2%)	4 (1%)	5 (1%)
Alcohol intake at recruitment*			
<5 g/day	248 (45%)	221 (40%)	235 (43%)
5–20 g/day	156 (28%)	173 (30%)	168 (30%)
≥20 g/day	152 (27%)	162 (30%)	150 (27%)
Continuous variables		**Median (5th–95th percentile)**	
Age at recruitment, y	56.9 (42–67)	56.9 (41.8–67.3)	56.6 (41.0–70.6)
Age at diagnosis, y	63.7 (49–75)	-	-
Time from blood draw to diagnosis, y	6.7 (1–12)	-	-
Plasma concentrations for components of the one-carbon metabolism			
Vitamin B2 (Riboflavin), nmol/L	13.9 (5.2–47.8)	14.4 (5.8–48.0)	13.2 (5.9–48.1)
Vitamin B6 (Pyridoxal 5′-phosphate), nmol/L	30.3 (13.0–89.0)	35.9 (14.6–122)	34.8 (14.3–97.0)
Folate (Vitamin B9), nmol/L	11.3 (3.9–32.2)	11.9 (4.5–36.6)	13.0 (5.1–35.6)

(*Table continues*)

Table 1. (Continued).

Continuous variables	RCC cases No. (%), n = 556	Matched controls No. (%), n = 556	Control group 2 No. (%), n = 553
Vitamin B12 (cobalamin), pmol/L	332 (192–582)	344 (194–588)	329 (178–523)
Methionine, μmol/L	24.1 (16.3–38.1)	25.0 (16.9–37.0)	25.1 (17.2–37.9)
Homocysteine, μmol/L	9.6 (6.2–18.4)	9.9 (6.1–17.3)	10.1 (6.4–18.4)

* Matching criteria were country, sex, and age at recruitment. EPIC = European Prospective Investigation into Cancer and Nutrition.

Table 2. Odds ratios of RCC for plasma concentrations of vitamins B2, B6, folate, B12, and homocycteine and methionine in the EPIC study

Quartile (range)	Cases, No. (%)	Controls, No. (%)	Odds ratio (95% confidence interval) Unadjusted conditional risk analysis* (n = 556/556)*	Conditional risk analysis adjusted for risk factors† (n = 556/556)†
Vitamin B2 (Riboflavin), nmol/L‡				
1 (2.57–9.83)	164 (30)	139 (25)	1.00 (referent)	1.00 (referent)
2 (9.84–14.3)	132 (24)	139 (25)	0.80 (0.57 to 1.12)	0.81 (0.56 to 1.16)
3 (14.4–22.2)	133 (24)	139 (25)	0.81 (0.58 to 1.13)	0.79 (0.56 to 1.13)
4 (22.2–416)	127 (23)	139 (25)	0.77 (0.55 to 1.09)	0.81 (0.56 to 1.17)
P_{trend}§			.13	.26
Vitamin B6 (Pyridoxal 5′-phosphate), nmol/L‡				
1 (5.95–25.4)	210 (38)	138 (25)	1.00 (referent)	1.00 (referent)
2 (25.4–35.9)	136 (25)	139 (25)	0.66 (0.48 to 0.91)	0.69 (0.49 to 0.98)
3 (35.9–51.9)	120 (22)	139 (25)	0.55 (0.39 to 0.77)	0.62 (0.43 to 0.89)
4 (51.9–436)	90 (16)	139 (25)	0.40 (0.28 to 0.57)	0.43 (0.29 to 0.64)
P_{trend}§			<.001	<.001
Vitamin B9 (Folate), nmol/L‡				
1 (0.20–8.40)	152 (27)	138 (25)	1.00 (referent)	1.00 (referent)
2 (8.41–11.8)	138 (25)	139 (25)	0.88 (0.62 to 1.24)	0.96 (0.66 to 1.40)
3 (11.9–17.3)	134 (24)	139 (25)	0.85 (0.59 to 1.22)	0.96 (0.65 to 1.43)
4 (17.3–109)	131 (24)	139 (25)	0.81 (0.55 to 1.20)	0.84 (0.55 to 1.27)
P_{trend}§			.08	.11
Vitamin B12 (Cobalamin), pmol/L‡				
1 (75.2–281)	154 (28)	138 (25)	1.00 (referent)	1.00 (referent)
2 (281–343)	148 (27)	139 (25)	0.96 (0.69 to 1.33)	0.96 (0.67 to 1.37)
3 (344–419)	137 (25)	139 (25)	0.88 (0.63 to 1.24)	0.87 (0.61 to 1.26)
4 (419–5000)	116 (21)	139 (25)	0.74 (0.52 to 1.05)	0.67 (0.46 to 0.99)
P_{trend}§			.12	.06
Methionine, μmol/L‡				
1 (2.1–21.48)	151 (27)	138 (25)	1.00 (referent)	1.00 (referent)
2 (21.5–25)	166 (30)	140 (25)	1.03 (0.74–1.44)	1.07 (0.75 to 1.52)
3 (25.01–28.94)	115 (21)	140 (25)	0.70 (0.49–1.01)	0.70 (0.47 to 1.03)
4 (28.95–71.4)	124 (22)	138 (25)	0.77 (0.53–1.11)	0.80 (0.53 to 1.19)
P_{trend}§			.06	.10
Homocysteine, μmol/L‡				
1 (3.68–8.03)	141 (25)	138 (25)	1.00 (referent)	1.00 (referent)
2 (8.04–9.87)	157 (28)	140 (25)	1.11 (0.79 to 1.56)	1.00 (0.69 to 1.43)
3 (9.88–11.9)	114 (21)	138 (25)	0.81 (0.56 to 1.17)	0.73 (0.49 to 1.09)
4 (12.0–64.9)	144 (26)	139 (25)	1.02 (0.69 to 1.51)	0.85 (0.56 to 1.30)
P_{trend}§			.55	.98

* Assessed by analyzing renal cell carcinoma (RCC) cases and their individually matched controls by conditional logistic regression, conditioning on individual case set. EPIC = European Prospective Investigation into Cancer and Nutrition.

† Assessed by analyzing RCC cases and their individually matched controls by conditional logistic regression after multiple imputation of missing covariate data, conditioning on individual case set, and adjusting for waist-to-hip ratio (quartiles defined among matched controls), hypertension (yes/no), educational attainment (four categories), smoking status (never/former/current), plasma cotinine (quartiles defined by the distribution for current smokers), alcohol intake at recruitment (g/day) and alcohol intake (ever/never). Case-control numbers only include those case sets where both the case and matched control had complete plasma measurements.

‡ Quartile cutoff points were determined based on the plasma level distribution of each biomarker for 556 individually matched controls.

§ P_{trend} assessed by the base 2 logarithm of plasma concentrations. All statistical tests were two-sided.

Table 3. Odds ratios of RCC for plasma concentrations of vitamin B6 for participants in the MCCS study

			Odds ratio (95% confidence interval)	
			Unadjusted conditional risk analysis*	Conditional risk analysis adjusted for risk factors†
Quartile (range)	Cases, No. (%)	Controls, No. (%)	(n = 144/144)*	(n = 144/144)†
Vitamin B6 (Pyridoxal 5′-phosphate), nmol/L ‡				
1 (5.31–25.4)	47 (33)	35 (24)	1.00 (referent)	1.00 (referent)
2 (25.4–35.9)	40 (28)	31 (22)	0.86 (0.44 to 1.67)	0.80 (0.40 to 1.61)
3 (35.9–51.9)	28 (19)	40 (28)	0.46 (0.22 to 0.95)	0.48 (0.23 to 1.01)
4 (51.9–435)	29 (20)	38 (26)	0.50 (0.25 to 1.01)	0.47 (0.23 to 0.99)
P_{trend}§			.02	.07

* Assessed by analyzing renal cell carcinoma (RCC) cases and their individually matched controls by conditional logistic regression, conditioning on individual case set. MCCS = Melbourne Collaborative Cohort Study; RCC = renal cell carcinoma.

† Assessed by analyzing RCC cases and their individually matched controls by conditional logistic conditioning on individual case set, and adjusting for waist-to-hip ratio (continuous), smoking status (never/former/current), plasma cotinine (continuous), and alcohol intake at recruitment (g/day).

‡ Quartile cutoff points were determined based on the plasma level distribution of each biomarker for 556 individually matched controls in the European Prospective Investigation into Cancer and Nutrition study

§ P_{trend} assessed by the base 2 logarithm of plasma concentrations. All statistical tests were two-sided.

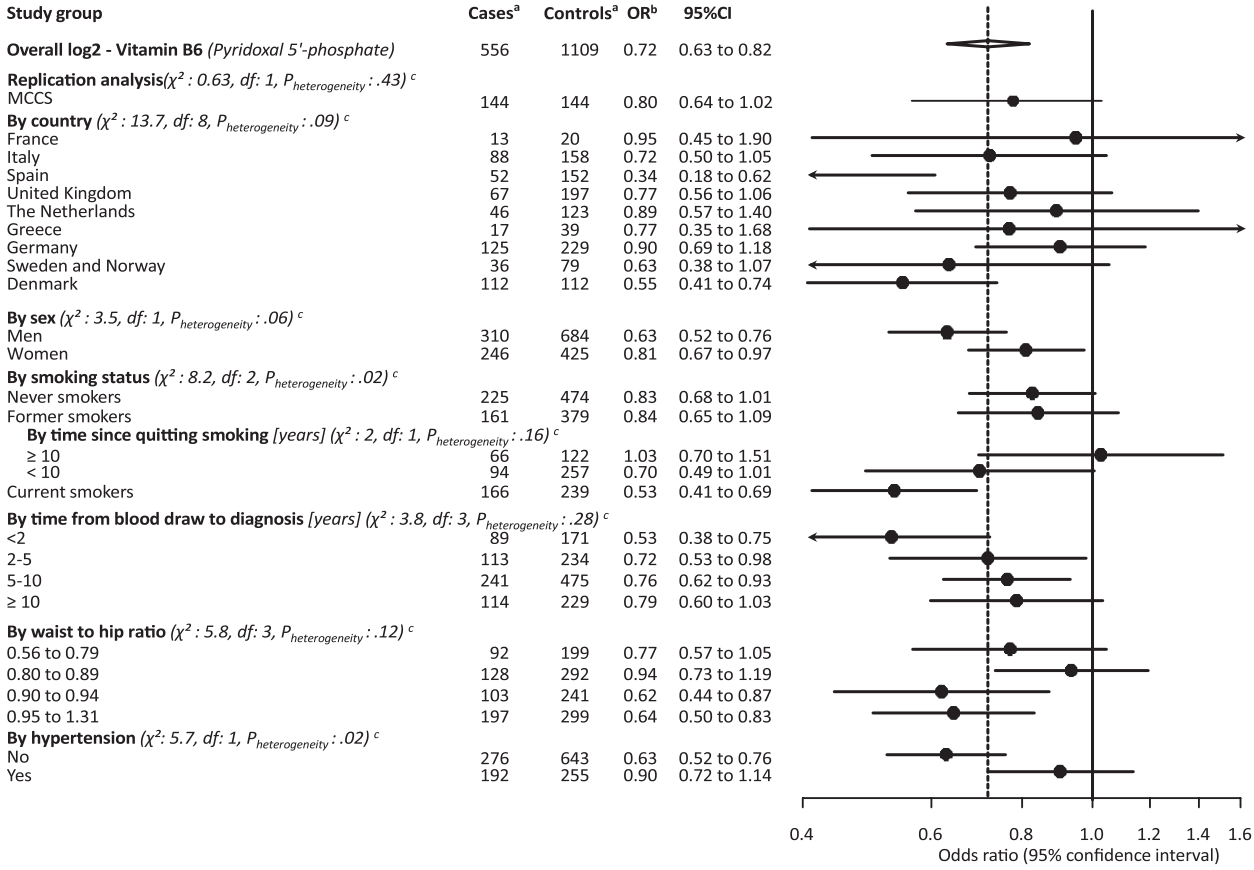

Figure 1. Forest plot showing overall odds ratios of renal cell carcinoma (RCC) for the base 2 logarithm of plasma vitamin B6 for the European Prospective Investigation into Cancer and Nutrition (EPIC) study, the Melbourne Collaborative Cohort Study (MCCS), as well as further stratified within the EPIC study alone. a, RCC cases and controls included in each stratified analysis (control group 2 was included). b, Odds ratios (ORs) were assessed by unconditional logistic regression by including the base 2 logarithm of plasma concentrations (ORs indicate relative risks of a doubling in plasma concentrations), and where relevant adjusted for age, sex, and country; the **black dots** indicate the ORs and the horizontal lines indicate the 95% confidence intervals. All statistical tests were two-sided. c, $P_{heterogeneity}$ indicates results of chi-square test assessing the null hypothesis of ORs being identical. The heterogeneity test for the replication analysis indicates any difference between the overall OR estimates of EPIC and MCCS. df = degrees of freedom.

analysis for potential confounders did not notably affect the HR estimates (Table 4). Model-based survival curves and Kaplan-Meier estimates are presented in Figure 2. Five-year survival proportions were 57% (95% CI = 50% to 64%) for RCC cases in the bottom 25% of plasma vitamin B6 and 73% (95% CI = 62% to 81%) for RCC cases in the upper 25% of plasma vitamin

Table 4. Hazard ratios of all-cause mortality for RCC cases for quartiles of plasma vitamins B2, B6, folate, B12, and homocysteine and methionine

Quartile (range)	Deceased*, No. (%)	Alive*, No. (%)	Person-years	Hazard ratio (95% confidence interval)	
				Minimally adjusted†	Adjusted for risk factors‡
Vitamin B2 (Riboflavin), nmol/L§					
1 (2.57–9.83)	50 (24)	111 (32)	744.1	1.00 (referent)	1.00 (referent)
2 (9.84–14.34)	52 (25)	75 (22)	602.8	1.23 (0.83 to 1.82)	1.32 (0.88 to 1.98)
3 (14.35–22.16)	48 (23)	85 (25)	528.9	1.01 (0.68 to 1.52)	1.09 (0.72 to 1.66)
4 (22.19–416.79)	55 (27)	73 (21)	501.4	1.19 (0.80 to 1.77)	1.38 (0.91 to 2.09)
P_{trend}‖				.75	.61
Vitamin B6 (Pyridoxal 5′-phosphate), nmol/L§					
1 (5.95–25.37)	90 (44)	116 (34)	890.4	1.00 (referent)	1.00 (referent)
2 (25.43–35.9)	51 (25)	83 (24)	576.5	0.87 (0.61 to 1.22)	0.87 (0.61 to 1.24)
3 (35.92–51.88)	37 (18)	84 (24)	530.8	0.64 (0.43 to 0.94)	0.66 (0.44 to 0.99)
4 (51.92–436.13)	27 (13)	61 (18)	379.5	0.57 (0.37 to 0.87)	0.59 (0.37 to 0.93)
P_{trend}‖				<.001	.004
Vitamin B9 (Folate), nmol/L§					
1 (0.2–8.4)	54 (26)	97 (28)	697.0	1.00 (referent)	1.00 (referent)
2 (8.41–11.84)	63 (31)	74 (22)	501.5	1.47 (1.02 to 2.13)	1.53 (1.04 to 2.24)
3 (11.86–17.25)	46 (22)	85 (25)	594.9	0.92 (0.61 to 1.37)	1.00 (0.66 to 1.51)
4 (17.27–109.35)	42 (20)	87 (25)	580.5	0.86 (0.57 to 1.30)	0.96 (0.62 to 1.47)
P_{trend}‖				.45	.88
Vitamin B12 (Cobalamin), pmol/L§					
1 (75.16–281.28)	61 (30)	90 (26)	638.9	1.00 (referent)	1.00 (referent)
2 (281.37–343.39)	57 (28)	90 (26)	600.5	0.94 (0.64 to 1.36)	0.96 (0.66 to 1.41)
3 (343.51–419)	46 (22)	88 (26)	595.5	0.75 (0.50 to 1.13)	0.75 (0.49 to 1.14)
4 (419.35–5000)	41 (20)	75 (22)	539.1	0.81 (0.54 to 1.21)	0.93 (0.61 to 1.41)
P_{trend}‖				.18	.58
Methionine, µmol/L§					
1 (2.1–21.48)	63 (31)	82 (24)	657.7	1.00 (referent)	1.00 (referent)
2 (21.5–25)	49 (24)	115 (33)	765.7	0.68 (0.46 to 0.99)	0.74 (0.50 to 1.10)
3 (25.01–28.94)	43 (21)	71 (21)	455.3	0.85 (0.57 to 1.27)	0.96 (0.63 to 1.44)
4 (28.95–71.4)	50 (24)	76 (22)	498.4	0.96 (0.65 to 1.41)	0.99 (0.67 to 1.47)
P_{trend}‖				.95	.67
Homocysteine, µmol/L§					
1 (3.68–8.03)	36 (18)	103 (30)	658.3	1.00 (referent)	1.00 (referent)
2 (8.04–9.87)	58 (28)	96 (28)	677.6	1.34 (0.88 to 2.05)	1.30 (0.85 to 2.00)
3 (9.88–11.94)	46 (22)	68 (20)	492.5	1.44 (0.92 to 2.25)	1.38 (0.88 to 2.16)
4 (11.95–64.88)	65 (32)	77 (22)	548.8	1.56 (1.02 to 2.40)	1.46 (0.94 to 2.25)
P_{trend}‖				.04	.11

* Vital status for RCC case at the last follow-up.

† Assessed by analyzing renal cell carcinoma (RCC) cases by Cox-proportional hazards regression, adjusting for country, sex, and age at diagnosis. RCC = renal cell carcinoma.

‡ Assessed by analyzing RCC cases by Cox-proportional hazards regression after multiple imputation of missing covariate data, adjusting for country, sex, and age at diagnosis, and further by quartiles of vitamin B6 (cutoffs defined in controls), hypertension (yes/no), waist-to-hip ratio (quartiles), educational attainment (four categories), smoking status (never/former/current), plasma cotinine (quartiles defined by the distribution for current smokers), alcohol intake at recruitment (g/day) and body mass index (three categories were defined: <25 kg/m2; 25–30 kg/m2, >30 kg/m2, in order to have a reasonable distribution of body mass index in each group). A corresponding sensitivity analysis is also provided in Supplementary Table 3 (available online) where participants with missing covariate data were excluded.

§ Quartile cutoff points were determined based on the plasma level distribution of each biomarker for 556 individually matched controls.

‖ P_{trend} assessed by the base 2 logarithm of plasma concentrations. All statistical tests were two-sided.

B6. Homocysteine also showed some association with all-cause mortality (P_{trend} = .04), but adjusting for potential confounders attenuated this association.

We replicated the results of vitamin B6 with RCC survival in MCCS (57 deaths in total), the all-cause mortality HR_{4vs1} estimate being 0.56 (95% CI = 0.27 to 1.17), and a doubling in vitamin B6 concentrations was associated with approximately 30% lower risk of death (HR_{log2} = 0.69, 95% CI = 0.52 to 0.93, P_{trend} = .02), and this estimate remained unchanged after adjusting for potential confounders (HR_{log2} = 0.69, 95% CI = 0.50 to 0.95, P_{trend} = .02). Similarly as in EPIC, for RCC cause-specific mortality in the MCCS (36 deaths) the association was strengthened (HR_{log2} = 0.54, 95% CI = 0.35 to 0.85, P_{trend} = .008).

Within the EPIC case series, stage at diagnosis was only available for 45% of cases, though the characteristics of cases with and without information on tumor stage were similar (Supplementary Table 5, available online). Within the subset of cases with stage available, an association between B6 and

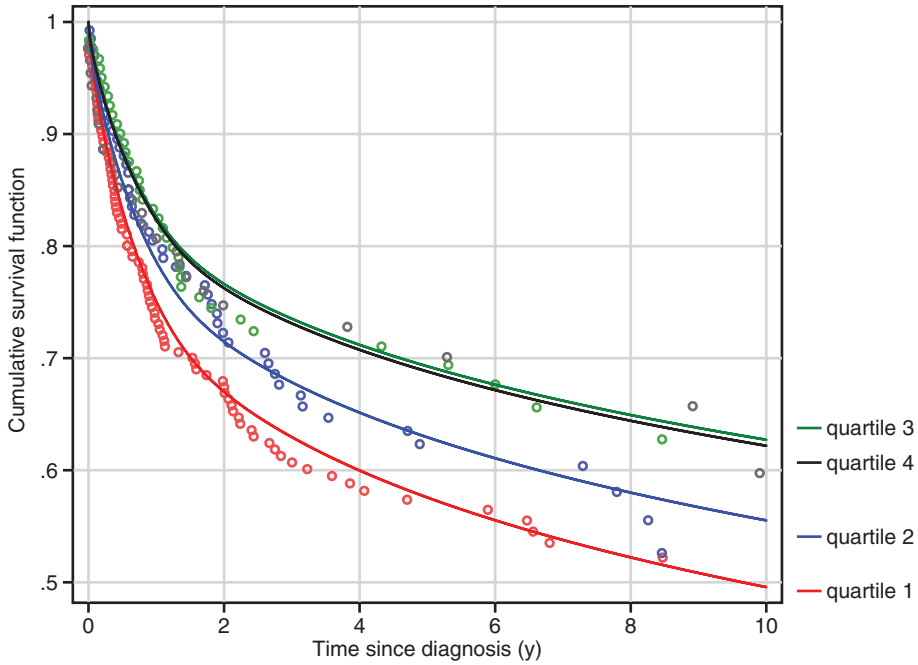

Figure 2. Cumulative survival curves of all-cause mortality in European Prospective Investigation into Cancer and Nutrition (EPIC) for study participants diagnosed with renal cell carcinoma (RCC) by quartiles of prediagnostic plasma vitamin B6. Cumulative survival curves of all-cause mortality for study participants diagnosed with RCC by prediagnostic plasma concentrations of vitamin B6 (quartiles based on the distribution for the controls). The **smooth lines** depict survival functions calculated from a flexible parametric survival model with proportional hazards for vitamin B6 quartiles. The **scattered points** are Kaplan-Meier estimates of the survival functions, evaluated at the time of death for each failure.

mortality was evident before adjusting for stage (HR_{log2} = 0.67, 95% CI = 0.50 to 0.91). As expected, adjusting for stage partly attenuated this association (HR_{log2} = 0.81, 95% CI = 0.60 to 1.10). Similarly in MCCS for which stage was available for 94% of cases, adjusting for stage attenuated the HR estimates (HR_{log2} = 0.81, 95% CI = 0.55 to 1.20).

Discussion

We investigated if differences in prediagnostic circulating concentrations of B vitamins and additional biomarkers of the one-carbon metabolism were associated with differences in risk of RCC and related mortality. We found that subjects with higher plasma concentrations of vitamin B6 had a clear decrease in risk of subsequent RCC in two separate cohorts, as well as improved survival following diagnosis.

To date, only one prospective study assessing circulating concentrations of Vitamin B6 and RCC has been published (11). This study was conducted on 224 RCC case-control pairs nested within the Alpha-Tocopherol, Beta-Carotene (ATBC) Cancer Prevention Study and did not, in contrast to our finding, report an association between vitamin B6 and risk. In our current EPIC study based on 556 prospectively collected case-control pairs, higher vitamin B6 concentrations were clearly associated with reduced RCC incidence, with subjects in the top quartile having approximately half the risk of developing the disease compared with subjects in the bottom quartile. When we externally replicated these EPIC results using the independent MCCS cohort from Melbourne, Australia, we observed nearly identical associations.

The ATBC study recruited participants within a homogeneous population of Finnish men for randomized supplementation with alpha-tocopherol, beta-carotene or placebo (23–25), whereas the EPIC study is a population-based observational study where recruitment took place across 10 Western European countries. The implications of these differences in study design and source populations are unclear, but may complicate direct comparisons between the studies (12). The biochemical measurements of vitamin B6 were also performed using different methodologies, ATBC applying the tyrosine decarboxylase assay and the EPIC and MCCS studies using liquid chromatography/tandem mass spectrometry. While both methods provide measurements of the active form of vitamin B6 (pyridoxal-5' phosphate), we note that ATBC controls had approximately 25% lower concentrations of vitamin B6 than did current smoking EPIC controls. It is unclear whether these differences explain the contrasting results, but several observations within the current study suggest that our findings were not because of chance or bias. For instance, the association between vitamin B6 concentrations and risk was nearly identical when comparing the cases with a separate control population, as well as when separately analyzing the independent cases and controls from the MCCS cohort. Taken together, these observations indicate that the association of vitamin B6 with risk is: 1) statistically robust and consistent across distinct European and Australian populations, 2) not because of random fluctuations in the case or control populations, and 3) not because of differential storage conditions or preanalytical treatment of cases and controls.

Several established risk factors for RCC could theoretically explain the association of vitamin B6 concentrations with risk, including tobacco smoking and obesity related factors, but the OR estimates were at most marginally affected when adjusting for these factors. Stratified analysis also showed that the decrease in

risk was evident up to 10 years after blood draw, suggesting that prediagnostic malignancies are unlikely to explain the results. We note that the association with risk was more prominent for smokers than never- and former smokers and for subjects without hypertension than for those with hypertension. While tobacco smoking is considered an established risk factor of RCC, it does not confer a particularly large increase in risk, current smokers having approximately a 50% risk increase compared with never smokers. That the effect estimate of the association between vitamin B6 and risk was twice that of being a current smoker would seem to rule out the possibility that tobacco exposure could be the underlying explanation for the observed association with vitamin B6. We were also able to control for circulating cotinine as a biomarker for recent tobacco exposure among current smokers. While adjustment for potential confounders did not affect the estimates substantially in either the EPIC or MCCS samples, we note that the set of potential confounders considered was the same in each study. As such, we cannot completely exclude the possibility that confounding by other unknown risk factors may partially account for the observed associations.

Our analysis also indicated that RCC cases with higher prediagnostic plasma B6 concentrations experience improved survival, the hazard ratio comparing the top and bottom quartiles being 0.57, an observation similar to the corresponding association with risk. While we mainly focused the survival analysis on all-cause mortality, an analysis of cause-specific mortality indicated that the association of B6 was driven by deaths caused by RCC. Indeed, no association between vitamin B6 and all-cause mortality was discernible in controls. Subsequent survival analyses among the cases from MCCS resulted in very similar hazard ratio estimates of all-cause mortality as in EPIC, thus providing an external replication that higher vitamin B6 was associated with improved survival. Similar to the EPIC analysis, the hazard ratio estimates were notably stronger in MCCS when considering cause-specific mortality of RCC.

We had limited information on stage at diagnosis for cases, being 45% complete in EPIC and 94% in MCCS, but adjusting for stage in these subsets provided similar and partly attenuated HR estimates. This result indicates that the survival benefit for subjects with higher vitamin B6 is at least partially mediated through stage. One interpretation of these findings is that lower B6 levels contribute to a greater risk of developing a more aggressive form of kidney cancer that presents with a later stage.

The current study was primarily initiated based on the hypothesis of one-carbon metabolism being important in renal cell carcinoma (9). However, multiple pathways implicated in cancer development are dependent on vitamin B6, examples of which include the tryptophan metabolism pathway, which is involved in immune function and inflammatory processes (26–28), as well as cell proliferation, angiogenesis, and oxidative stress (29), the latter being particularly relevant in smoking-related cancers. Our results should therefore not be taken as evidence for a causal role of dietary intake of vitamin B6 in RCC aetiology (30). Indeed, dietary intake of vitamin B6 is not strongly related to circulating vitamin B6 levels and has not been associated with RCC risk (31,32). This observation would seem to warrant further research on lifestyle and metabolic determinants of circulating vitamin concentrations. Indeed, given the number of enzymatic reactions that are dependent on vitamin B6—over 100 having been identified to date (33,34)—adopting a more agnostic approach, such as metabolomics-based methodologies, may shed further light on the metabolic pathways involving vitamin B6 in the pathogenesis of renal cell carcinoma. Such an approach might also benefit from the use of multiple assessments per research participant in order to examine the extent to which within-person variability affects estimated associations between metabolites and risk

High circulating concentrations of vitamin B6 were strongly associated with decreased risk of RCC. Our results also suggest a potentially important association with survival after RCC diagnosis. Further elucidating the metabolic pathways underlying this relationship and the extent to which it can be influenced by changes in lifestyle may suggest preventive strategies for RCC and other cancers, as well as improved cancer survival.

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Funding

The World Cancer Research Fund (UK) supported the biochemical analyses for the European Prospective Investigation into Cancer and Nutrition (EPIC) study. The funding organization had no role in design and conduct of the study, collection, management, analysis, or interpretation of the data, preparation, review, or approval of the manuscript. The work undertaken by DCM that was reported in this paper was done during the tenure of an International Agency for Research on Cancer (IARC)–Australia Postdoctoral Fellowship from the International Agency for Research on Cancer, supported by the Cancer Council Australia. Dr. Ueland reports that he is a member of the steering board of the nonprofit Foundation to Promote Research Into Functional Vitamin B12 Deficiency.

The EPIC study has been supported by the Europe Against Cancer Program of the European Commission (SANCO); Deutsche Krebshilfe, Deutsches Krebsforschungszentrum, German Federal Ministry of Education and Research; Danish Cancer Society; Health Research Fund (FIS) of the Spanish Ministry of Health, Spanish Regional Governments of Andalucia, Asturias, Basque Country, Murcia and Navarra; Catalan Institute of Oncology, Spain; the ISCIII of the Spanish Ministry of Health (RETICC DR06/0020); Cancer Research UK; Medical Research Council, UK; Greek Ministry of Health; Stavros Niarchos Foundation; Hellenic Health Foundation; Italian Association for Research on Cancer (AIRC); Italian National Research Council, Fondazione-Istituto Banco Napoli, Italy; Associazione Italiana per la Ricerca sul Cancro-AIRC-Milan; Compagnia di San Paolo; Dutch Ministry of Public Health, Welfare and Sports; World Cancer Research Fund; Swedish Cancer Society; Swedish Scientific Council; Regional Government of Västerbotten, Sweden; NordForsk (Centre of excellence programme HELGA), Norway; French League against Cancer (LNCC), France; National Institute for Health and Medical Research (INSERM), France; Mutuelle Générale de l'Education Nationale (MGEN), France; 3M Co, France; Gustave Roussy Institute (IGR), France; and General Councils of France.

Notes

Author contributions: MJ and PB initiated, acquired the main funding, and designed this investigation, with additional funding for the Melbourne Collaborative Cohort Study (MCCS) analysis provided by GS. PMU, SEV, and ØM led the laboratory analysis. MJ led the statistical analysis of EPIC data with important contributions from AF, DCM. JKB and DCM led the statistical analysis of MCCS data. MJ drafted the first version of the manuscript with important contributions from AF, DCM, JKB, PMU, GS, and PB. All authors were involved with collection of data, data interpretation, critical revision of the manuscript, and approval of the final version. MJ and PB had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. ER is the overall coordinator of the EPIC study, which he designed and implemented in collaboration with the main investigators in the collaborating centers.

We declare that we have no conflicts of interest.

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