One-carbon metabolism biomarkers and risk of urothelial cell carcinoma in the European prospective investigation into cancer and nutrition

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Published associations between dietary folate and bladder cancer risk are inconsistent. Biomarkers may provide more accurate measures of nutrient status. This nested case–control analysis within the European Prospective Investigation into Cancer and Nutrition (EPIC) investigated associations between pre-diagnostic serum folate, homocysteine, vitamins B6 and B12 and the risk of urothelial cell carcinomas of the bladder (UCC). A total of 824 patients with newly diagnosed UCC were matched with 824 cohort members. Serum folate, homocysteine, and vitamins B6 and B12 were measured. Odds ratios (OR) and 95% confidence intervals (CI) for total, aggressive, and non-aggressive UCC were estimated using conditional logistic regression with adjustment for smoking status, smoking duration and intensity, and other potential confounders. Additionally, statistical interaction with smoking status was assessed. A halving in serum folate concentrations was moderately associated with risk of UCC (OR: 1.18; 95% CI: 0.98–1.43), in particular aggressive UCC (OR: 1.34; 95% CI: 1.02–1.75; p-heterogeneity = 0.19). Compared to never smokers in the highest quartile of folate concentrations, this association seemed only apparent among current smokers in the lowest quartile of folate concentrations (OR: 6.26; 95% CI: 3.62–10.81, p-interaction = 0.07). Dietary folate was not associated with aggressive UCC (OR: 1.26; 95% CI: 0.81–1.95; p-heterogeneity = 0.14). No association was observed between serum homocysteine, vitamins B6 and B12 and risk of UCC. This study suggests that lower serum folate concentrations are associated with increased UCC risk, in particular aggressive UCC. Residual confounding by smoking cannot be ruled out and these findings require confirmation in future studies with multiple measurements.

What's new?

Results of studies on dietary folate and bladder cancer risk are inconsistent. This nested case-control analysis within the European Prospective Investigation into Cancer and Nutrition is one of the first prospective study investigating associations between pre-diagnostic serum folate, homocysteine, vitamins B6 and B12 and the risk of urothelial cell carcinomas of the bladder (UCC). This study suggests that lower serum folate concentrations are associated with increased risk of UCC, in particular aggressive UCC.

Introduction

Folate and related nutrients may influence cancer initiation and progression through their role in the one-carbon metabolism pathway, which is necessary for DNA synthesis, repair, and methylation.[1] They are present in a wide range of foods, including fruit and vegetables. There is limited suggestive evidence that fruit and vegetable consumption is inversely associated with bladder cancer risk.[2] Case–control studies have

shown that intake of folate and other B-vitamins may also reduce risk of bladder cancer, but results from prospective studies are inconsistent.[3] This inconsistency may partly be due to misclassification of self-reported intake. Thus far, only two studies used circulating blood concentrations of folate, which reflect both dietary and supplemental intakes. A case–control study found an inverse association for plasma folate concentrations and bladder cancer risk,[4] but may be biased by changes in blood concentrations after diagnosis. A recent nested case–control study investigating pre-diagnostic plasma folate concentrations found no association with bladder cancer risk.[5]

In addition to folate status assessment methods, the heterogeneous nature of bladder cancer may contribute to inconsistent study results. Urothelial cell carcinoma (UCC), the most common morphological type of bladder cancer, varies in natural history. Stratification into different subgroups according to stage and grade may help to identify risk factors involved in different UCC pathways. It has been suggested that associations with cigarette smoking and employment in a high-risk occupation, the most well-known risk factors for UCC,[6] are stronger for bladder tumours with a higher stage and grade.[7–9] However, other studies did not find different associations by tumour stage.[10,11] Previous findings from our group have shown that plasma concentrations of carotenoids may particularly influence the risk of bladder tumours with a higher stage and grade.[12] Interestingly, some studies showed that global DNA methylation may also be differently associated with UCC subtypes, with higher methylation levels associated with a reduced risk of lower stage and grade tumours[13,14] and intermediate methylation levels with a reduced risk of higher stage and grade tumours.[14] Thus, the unavailability of folate-derived methyl groups may influence the risk of UCC through altered DNA methylation.

This nested case–control analysis within the European Prospective Investigation into Cancer and Nutrition (EPIC) is one of the first prospective studies that investigated associations between pre-diagnostic serum compounds involved in one-carbon metabolism (folate, homocysteine, vitamin B6, vitamin B12) and the risk of UCC.

Subjects and Methods

Study population and data collection

The design and methods of the EPIC study have been described in detail previously.[15,16] Briefly, EPIC is a cohort study comprising more than half a million people recruited in 10 European countries. Between 1992 and 2000, standardised questionnaires on diet, lifestyle questionnaires, and medical history were collected at enrolment.[15–17] Intakes of dietary folic acid and vitamins B2, B6, and B12 were estimated using the updated EPIC Nutrient Data Base, obtained after standardisation from country-specific food composition tables.[17] Study centres collected and stored blood samples according to a standardised protocol.[15] Biological samples (74% of all respondents donated samples) are stored at the International Agency for Research on Cancer (IARC, Lyon, France) in −196 °C liquid nitrogen for all countries except

Denmark (−150 °C, nitrogen vapour) and Sweden (−80 °C, freezers). While protected from light after blood donation, the blood samples from Oxford and Norway were exposed to ambient temperatures for up to 48 h. As B-vitamin concentrations are partly degraded by such handling, the samples from these centres were excluded from the present analyses.[15,16] All participants gave written informed consent, and the study was approved by the Institutional Review Board of IARC and the local ethics committees in the participating countries.

Nested case–control design and selection of participants

Ascertainment of newly diagnosed bladder cancer patients has been described previously.[12] Participants were followed from study entry until a first primary bladder cancer diagnosis (code C67 according to the ICD–Oncology, third edition), end of follow-up (between 2002 and 2005 in different countries), last known contact date, cancer diagnosis, or death. Only (papillary) urothelial cell carcinomas (UCC morphology codes 8,120 and 8,130) were included in the analyses, as these comprise more than 90% of bladder cancers. Pathology reports of UCC cases were collected from each centre to obtain information on stage and differentiation grade of the tumour. Stage T1 and higher, carcinoma *in situ*, or WHO grade 3 carcinomas were classified as aggressive UCCs, whereas stage Ta grades 1 or 2 were classified as non-aggressive UCCs.[18] Ninety-two percent of the cases could be classified as aggressive or non-aggressive UCC.

Each case was matched to one control by incidence density sampling from all cohort members alive without a cancer diagnosis of any kind (except non-melanoma skin cancer) at the time of diagnosis of the case. Controls were randomly selected from the population at risk at the time of diagnosis of the case.[19] Matching criteria were sex, age at time of enrolment (± 3 years), study centre, date of blood collection (± 3 months), time of day of blood collection (± 2 h) and fasting status at the time of blood collection (< 3, 3–6, and > 6 h since last meal). Matched controls were unavailable for 11 cases and these individuals were excluded from analyses. Case sets including participants with missing blood samples (n = 110), no information on follow-up (n = 6) or smoking history (n = 15), from Oxford or Norway centre (n = 49), or including cases with other morphology codes than 8,120 and 8,130 (n = 78) were also excluded. Our study included 824 pairs of first primary UCC cases and their matched controls.

Laboratory assays

Serum samples were analysed at the National Institute for Public Health and the Environment (Bilthoven, The Netherlands) for folate (vitamin B9) and vitamin B12 (cobalamin) by using an Access-2 immunoanalyzer (Beckman-Coulter). Vitamin B6 (pyridoxal-5-fosfate) was determined by reversed-phase HPLC analysis with fluorescence detection using a kit of Chromsystems (Munich, Germany). Vitamin B2 (riboflavin) could not be assayed because the amount of available blood was insufficient. The HPLC equipment was from Varian Association

(Middelburg, the Netherlands) equipped with a fluorescence detector from Jasco (Separations, Hendrik-Ido-Ambacht, The Netherlands). Homocysteine concentrations were determined with an enzyme cycling assay (Dialab, Neudorf, Austria) on an LX20-Pro autoanalyser (Beckman-Coulter, Woerden, The Netherlands). For quality control, one control sample with concentrations similar to the mean concentrations in controls was added to each batch of samples to assess interbatch reproducibility. There were 21 batches. Coefficients of variation for these quality-control samples were 6.1% for folate, 3.4% for homocysteine, 3.5% for vitamin B6, and 7.2% for vitamin B12. To minimise the influence of batch-to-batch variation, cases were analysed in the same analytic batch as their matched controls. Laboratory technicians were blinded to the case–control status of the samples, and biochemical analyses were done in one laboratory, with avoidance of between-laboratory method variability.

Statistical analysis

Odds ratios (OR) and 95% confidence intervals (95% CI) for UCC risk in relation to serum concentrations and dietary intakes were calculated using conditional logistic regression models stratified by the case–control set.[20] ORs were calculated using quartiles with cutoffs based on the distribution of serum and/or dietary intake levels of folate, homocysteine, vitamins B2, B6 and B12 in control subjects. We derived probability values for a linear trend across quartiles from regression models by using the median log2-transformed serum and/or dietary levels within quartiles as a continuous variable.[21] The data were also analysed as log2-transformed continuous variables. The ORs of these log2-transformed variables correspond to the increase in UCC risk with the halving of the serum concentration or dietary intake. To investigate departure from linearity, we also included a quadratic term for each log2-transformed serum and dietary intake level and tested for a quadratic trend using the likelihood ratio test.

All models were adjusted for smoking status (never, former, current), duration of smoking (years), and lifetime intensity of smoking (cigarettes/day). The final models were additionally adjusted for energy intake (continuous), consumption of processed or red meat (continuous), alcohol intake (continuous), physical activity (inactive, moderately inactive, moderately active, active, unknown), BMI (continuous), and educational level (primary school or less, technical-professional school, secondary school, university, unspecified). Occupational history was not included in the final models, since it was only available for a subset of the population and did not affect the β estimates of serum and/or dietary levels within this subset. We also tested whether further adjustment for the other B-vitamins and homocysteine changed the risk estimates. As previous findings in the same study population suggest a reduced UCC risk with higher concentrations of the sum of plasma carotenoids[12] and an increased UCC risk with plasma α-tocopherol (data not shown), we also tested further adjustment for these micronutrients.

Polytomous conditional logistic regression stratified by the case–control set was used to assess the association of serum concentrations and dietary intakes with aggressive and non-aggressive UCC as separate endpoints.[22] Statistical heterogeneity of associations across these subtypes was assessed with a likelihood ratio test, comparing a model in which the association could vary by subtype *versus* a model assuming the same association across subtypes.

Because smoking may not only confound but also modify the association,[23,24] joint effects were determined for quartiles of serum one-carbon metabolism biomarkers in combination with smoking status (never, former, current) in relation to UCC risk. The combined category of high serum concentrations with never smoking was chosen as reference. Statistical interaction on a multiplicative scale was tested by introducing a product term between serum one-carbon metabolism biomarkers (quartiles) and smoking status. Similar models were used to test for interactions with BMI,[25] gender and alcohol intake.[26] Joint effects of folate, homocysteine, vitamin B6 and vitamin B12 were also conducted to evaluate whether the measured analytes interacted with each other. Analyses of joint effects were performed unadjusted and adjusted for smoking status, duration of smoking and lifetime intensity of smoking. To evaluate whether preclinical disease may have influenced the results, analyses were repeated after exclusion of cases along with their matched controls who were diagnosed within 2 years after recruitment (n = 136 pairs).

All statistical analyses were performed with SAS software (version 9.2, SAS Institute Inc). For all analyses, two-sided $p < 0.05$ was considered statistically significant.

Results

Baseline characteristics of cases diagnosed with UCC and their matched controls are shown in Table 1. UCC cases were more likely to smoke, were slightly less educated, reported a higher intake of alcohol and red and processed meat, and consumed less fruit and vegetables. Spearman correlations between the individual serum biomarkers and between the serum biomarkers and the dietary intake levels of the B-vitamins were statistically significant but generally weak, ranging from −0.23 to 0.38.

A halving in serum folate concentrations was moderately associated with increased risk of UCC (OR: 1.18; 95% CI: 0.98–1.43) (Table 2). When stratified by prognostic subgroups of UCC, a halving in serum folate was positively associated with aggressive UCC (OR: 1.34; 95% CI: 1.02–1.75). Serum folate was not associated with non-aggressive UCC (OR: 1.03; 95% CI: 0.78–1.37), and no statistically significant heterogeneity by tumour subtype was observed (*p*-heterogeneity = 0.19). Quartile analyses showed similar results. The associations of folate were similar for models only adjusted for smoking (Supporting Information Table S1) and remained after adjusting the final models for the blood concentrations of homocysteine, vitamin B6, vitamin B12, α-tocopherol or the sum of

Table 1. Baseline characteristics of urothelial cell carcinoma cases and their matched controls

	Cases (n = 824)	Controls[9] (n = 824)
General characteristics		
Men, n(%)	611 (74)	611 (74)
Women, n(%)	213 (26)	213 (26)
BMI (kg/m^2)	26.6 ± 4.0[1]	26.4 ± 3.8
Physical active[2] (%)	42	43
Age at recruitment (y)	58.4 ± 7.4	58.4 ± 7.4
Smoking status		
Never smokers (n, %)	156 (19)	316 (38)
Former smokers (n, %)	294 (36)	287 (35)
Lifetime number of cigarettes (cig/day)	10.2 ± 10.2	9.9 ± 10.2
Smoking duration (y)	25.3 ± 13.6	21.8 ± 12.9
Age at start smoking (y)	17.9 ± 5.6	19.0 ± 6.6
Time since quitting smoking (y)	13.4 ± 11.4	16.8 ± 11.8
Current smokers (n, %)	374 (45)	221 (27)
Lifetime number of cigarettes (cig/day)	14.1 ± 9.3	11.7 ± 8.8
Smoking duration (y)	38.7 ± 10.0	36.1 ± 11.6
Age at start smoking (y)	17.9 ± 5.9	19.6 ± 7.9
Exposure to occupational carcinogens (n, %)[3]		
Heavy metals	168 (12)	159 (11)
Aromatic amines	132 (9)	115 (8)
Polycylic Aromatic Hydrocarbons (PAH)	115 (8)	94 (7)
Environmental tobacco smoke	37 (3)	38 (3)
Educational level (n, %)		
None or primary school	387 (47)	338 (41)
Technical/professional school	200 (24)	230 (28)
Secondary school	90 (11)	86 (10)
University degree	142 (17)	159 (20)
Not specified	5 (1)	11 (1)
Serum concentrations micronutrients (median, 5th - 95th percentile)		
Folate (nmol/L)	14.9 (7.2–31.4)[4]	16.0 (7.9–34.9)
Homocysteine (μmol/L)	16.6 (9.4–26.3)	16.0 (8.7–25.6)
Vitamin B6 (μg/L)	37.5 (15.8–124.0)	40.1 (15.6–122.9)
Vitamin B12 (pmol/L)	275 (140–547)	270 (140–492)
Dietary factors		
Total energy (kcal/d)	2,266.1 ± 665.4	2,288.6 ± 716.3
Energy from fat (kcal/d)	784.9 ± 285.8	790.2 ± 296.9
Energy from non-fat (kcal/d)	1,481.2 ± 446.2	1,498.4 ± 484.5
Alcohol consumption (g/d)	19.2 ± 22.7	17.6 ± 21.4
Red and processed meat (g/d)	96.9 ± 56.7	95.2 ± 61.7
Fresh fruits (g/d)	195.5 ± 163.7	217.4 ± 189.9
Total vegetables (g/d)	169.5 ± 142.6	176.9 ± 125.2
Dietary folate intake (μg/d)	291.2 ± 106.9	302.6 ± 132.6
Dietary vitamin B2 (mg/d)	2.0 ± 0.63	2.04 ± 0.78
Dietary vitamin B6 intake (mg/d)	1.92 ± 0.80	1.91 ± 0.77
Dietary vitamin B12 intake (μg/d)	7.9 ± 6.0	7.4 ± 4.5

(Continues)

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Table 1. Baseline characteristics of urothelial cell carcinoma cases and their matched controls (Continued)

	Cases (n = 824)	Controls[9] (n = 824)
Cases only		
Age at diagnosis	63.2 ± 7.7	-
Time from blood draw to diagnosis (y)	4.6 ± 2.8	-
Urothelial cell carcinomas (n,%)[5]		
Aggressive[6]	390 (47)	-
Non-aggressive[7]	374 (45)	-
Unknown[8]	60 (8)	-

[1]Mean ± SD (all such values).
[2]Cambridge Physical Activity Index incorporates occupational and nonoccupational physical activity.
[3]Data were not available for Umea (Sweden), Norway, Naples (Italy), Utrecht (Netherlands), and France.
[4]Median; 5th – 95th percentile in parentheses (all such values).
[5]Includes urothelial cell papillomas and carcinomas (morphology codes 8,120 and 8,130, and behaviour coded as uncertain whether benign or malignant, carcinoma *in situ*, and/or malignant) but excludes inverted papillomas (8,121/1).
[6]Includes all stage T1 or higher, carcinoma *in situ*, or WHO 1973 grade 3 carcinomas (including Ta grade 3).
[7]Includes all stage Ta grade 1 or Ta grade 2 carcinomas.
[8]Sixty urothelial cell carcinomas could not be classified as aggressive or nonaggressive urothelial cell carcinomas because of lack of information on stage or grade.
[9]Controls were matched to cases by sex, age at baseline, study centre, date and time of blood collection, and fasting status.

carotenoids (data not shown). Serum concentrations of homocysteine, vitamin B6, or vitamin B12 were not related to UCC risk (Table 2; Supporting Information Table S1). No evidence for a quadratic relationship was found (data not shown). The positive and null associations persisted after exclusion of cases diagnosed during the first 2 years of follow-up (data not shown).

For a halving in dietary folate levels, an increased risk was seen for aggressive UCC in the smoking adjusted model (OR: 1.45; 95% CI: 1.03–2.04; Supporting Information Table 2) which was attenuated in the fully adjusted model (OR: 1.26; 95% CI: 0.81–1.95; Table 3). No significant heterogeneity by tumour subtype was observed (p-heterogeneity = 0.07 and 0.14, respectively). Intakes of dietary vitamin B2, vitamin B6 and vitamin B12 were not associated with UCC risk (Table 3). There was some indication that the association of dietary folate and dietary vitamin B6 may be non-linear ($P \leq 0.05$ for a model with *vs.* without a quadratic term).

We further studied whether there was statistical interaction between serum folate and smoking status in relation to UCC risk. The elevated risk of UCC seemed only apparent for current smokers with lowest folate concentrations compared to never smokers with highest folate concentrations in analyses unadjusted (OR for lowest *vs.* highest quartile (OR_{Q1-Q4}): 6.26; 95% CI: 3.62–10.81; p-interaction = 0.07; Table 4) and adjusted (OR_{Q1-Q4}: 2.02; 95% CI: 1.07–3.82; p-interaction = 0.11; Supporting Information Table 3) for smoking duration and intensity. In all other groups, risks were not significantly elevated. These data suggest that there is statistical interaction between serum folate and smoking status in relation to risk of UCC. No interaction with smoking status was observed for the associations of homocysteine, vitamin B6, and vitamin B12 with risk of UCC. Interactions on a multiplicative scale between serum folate and these three biomarkers were not significant (p-interaction>0.05). Further, no statistical interaction

was observed between all four biomarkers and alcohol intake, gender or BMI in relation to UCC risk (p-interaction>0.05) (data not shown).

Discussion

In this large prospective study, lower serum folate concentrations were associated with an increased risk of UCC, in particular aggressive UCC. The positive association with lower serum folate concentrations seemed only apparent in current smokers. Dietary folate intake was associated with an increased risk of aggressive UCC in the smoking adjusted models but this association was attenuated in the fully adjusted models. Other nutrients involved in the one-carbon metabolism pathway, i.e. serum homocysteine, vitamin B6, or vitamin B12, were not associated with UCC risk.

Previous findings of the relation between dietary intake of B-vitamins and the risk of bladder cancer have yielded inconsistent results. A meta-analysis observed an inverse association between folate intake and bladder cancer risk in six case–control studies (OR = 0.73; 95% CI 0.57–0.89), but not in seven cohort studies (relative risk = 0.96; 95% CI 0.81–1.10) (3). Also a recent cohort study found no association of dietary intake of folate and other B-group vitamins with UCC risk, and did not observe heterogeneity across UCC subtypes.[27] Self-reported dietary folate intake may be a less accurate measure of folate status than blood folate concentrations, which reflect both dietary and supplemental intakes and is directly related to bioavailability. One case–control study from Taiwan with 171 bladder cancer cases reported a decreased bladder cancer risk with higher plasma folate concentrations (4). A recent nested case–control study from Australia with 363 UCC cases found no association between pre-diagnostic plasma folate concentrations and UCC risk (5).

Table 2. Serum concentrations of folate, homocysteine, vitamin B6 and B12 and the risk of urothelial cell carcinomas[1]

	No. of cases/controls	Total UCC[2]	No. of cases/controls	Aggressive UCC[2]	No. of cases/controls	Non-aggressive UCC[2]
Folate (nmol/L)[3]						
≤ 11.92	245/198	1.37 (0.97–1.92)	115/83	1.66 (1.02–2.70)	116/106	0.97 (0.58–1.64)
11.95–16.02	205/199	1.30 (0.94–1.79)	91/100	1.23 (0.76–2.00)	98/85	1.24 (0.75–2.04)
16.03–21.32	174/202	1.04 (0.76–1.42)	86/91	1.12 (0.70–1.79)	74/98	0.81 (0.50–1.32)
≥ 21.33	175/200	1.00	84/102	1.00	77/76	1.00
p for trend[4]		0.04		0.04		0.69
Continuous after log2-transformation[5]		1.18 (0.98–1.43)		1.34 (1.02–1.75)		1.03 (0.78–1.37)
p for heterogeneity[6]					0.19	
Homocysteine (µmol/L)[7]						
≤ 13.32	177/200	0.80 (0.57–1.15)	72/85	0.80 (0.48–1.34)	88/92	1.02 (0.60–1.72)
13.33–15.94	176/198	0.74 (0.54–1.03)	79/92	0.65 (0.41–1.03)	83/91	0.90 (0.54–1.50)
15.95–19.01	215/202	0.96 (0.72–1.28)	110/113	0.79 (0.53–1.18)	92/76	1.38 (0.87–2.20)
≥ 19.05	232/200	1.00	119/90	1.00	99/103	1.00
p for trend[4]		0.16		0.31		0.80
Continuous after log2-transformation[5]		0.85 (0.65–1.11)		0.84 (0.56–1.25)		0.87 (0.60–1.26)
p for heterogeneity[6]					0.89	
Vitamin B6 (µg/L)[8]						
≤ 27.96	226/186	1.11 (0.79–1.56)	95/89	1.00 (0.62–1.63)	115/82	1.17 (0.71–1.94)
28.04–39.86	184/187	1.13 (0.81–1.58)	91/76	1.50 (0.92–2.45)	78/100	0.79 (0.48–1.31)
39.96–58.41	173/187	1.03 (0.75–1.43)	81/89	1.04 (0.65–1.67)	75/83	0.90 (0.55–1.47)
≥ 59.24	166/189	1.00	82/95	1.00	76/79	1.00
p for trend[4]		0.48		0.72		0.58
Continuous after log2-transformation[5]		0.96 (0.85–1.09)		0.94 (0.78–1.13)		0.96 (0.80–1.15)
p for heterogeneity[6]					0.85	
Vitamin B12 (pmol/L)[9]						
≤ 214	204/195	0.94 (0.69–1.28)	99/99	0.86 (0.54–1.37)	88/81	0.92 (0.58–1.44)
215–270	182/204	0.84 (0.61–1.15)	90/99	0.77 (0.48–1.23)	78/89	0.87 (0.54–1.39)
271–346	207/201	1.02 (0.76–1.37)	94/88	1.03 (0.68–1.57)	95/98	0.92 (0.59–1.44)
≥ 347	212/205	1.00	97/94	1.00	105/98	1.00
p for trend[4]		0.53		0.40		0.67
Continuous after log2-transformation[5]		0.87 (0.71–1.05)		0.88 (0.67–1.17)		0.81 (0.61–1.09)
p for heterogeneity[6]					0.69	

[1]The Total UCC group includes urothelial cell papillomas and carcinomas (morphology codes 8,120 and 8,130, and behaviour coded as uncertain whether benign or malignant, carcinoma *in situ*, and/or malignant) but excludes inverted papillomas (8,121/1). The Aggressive UCC category includes all stage T1 or higher, carcinoma *in situ*, or WHO 1973 grade 3 carcinomas (including Ta grade 3). The group Non-aggressive UCC category includes all stage Ta grade 1 or Ta grade 2 carcinomas.
[2]All values are odds ratios; 95% CIs in parentheses. Analyses were matched for age at blood collection, study centre, sex, date and time of blood collection, and fasting status and further adjusted for smoking status, duration and intensity of smoking, energy intake, red meat intake, processed meat intake, alcohol intake, physical activity, BMI, educational level.
[3]For 46 cases and/or controls information is lacking on serum folate.
[4]Test for trend was performed using the median log2 value of each quartile.
[5]Values were derived from adjusted models as described in footnote 2 for risk associated with a halving in serum concentration.
[6]Likelihood ratio test of heterogeneity, testing for a common association across subtypes.
[7]For 44 cases and/or controls information is lacking on serum homocysteine.
[8]For 146 cases and/or controls information is lacking on serum vitamin B6.
[9]For 34 cases and/or controls information is lacking on serum vitamin B12.

Folate plays an important role in DNA synthesis, repair, and methylation.[1] Low plasma folate concentrations may increase the risk of UCC and other cancers by inducing DNA hypomethylation, potentially leading to dysregulation of proto-oncogenes and tumour suppressor genes.[1] UCC is a complex disease with etiologic heterogeneity and appears to occur *via* distinct molecular pathways. Interestingly, besides a differential association of global DNA methylation with UCC subtypes,[13,14] a high-throughput DNA methylation analysis of UCC tumours also revealed distinct methylation patterns.[28] Non-aggressive

Table 3. Dietary folate, vitamins B2, B6 and B12 and the risk of urothelial cell carcinomas[1]

	No. of cases/controls	Total UCC[2]	No. of cases/controls	Aggressive UCC[2]	No. of cases/controls	Non-aggressive UCC[2]
Dietary folate (µg/d)						
≤ 226.74	225/204	1.18 (0.76–1.84)	98/80	1.42 (0.78–2.59)	110/107	1.00 (0.56–1.81)
227.40–282.19	216/206	1.17 (0.81–1.69)	104/98	1.25 (0.76–2.06)	99/98	1.10 (0.65–1.84)
282.46–350.55	201/206	1.19 (0.86–1.65)	98/107	1.28 (0.80–2.03)	85/83	1.12 (0.68–1.83)
≥ 350.71	180/206	1.00	88/103	1.00	80/86	1.00
p for trend[3]		0.47		0.27		0.99
Continuous after log2-transformation[4]		1.03 (0.73–1.47)		1.26 (0.81–1.95)		0.85 (0.53–1.36)
p for heterogeneity[5]					0.14	
Dietary vitamin B2 (mg/d)						
≤ 1.43	233/205	1.15 (0.76–1.75)	110/103	1.12 (0.63–1.97)	103/86	1.11 (0.63–1.96)
1.41–1.78	181/206	0.87 (0.61–1.25)	96/88	1.15 (0.71–1.88)	71/104	0.63 (0.37–1.07)
1.78–2.25	207/205	1.10 (0.80–1.51)	92/90	1.22 (0.77–1.94)	100/97	1.02 (0.64–1.63)
≥ 2.25	201/206	1.00	90/107	1.00	100/87	1.00
p for trend[3]		0.68		0.69		0.87
Continuous after log2-transformation[4]		0.91 (0.67–1.25)		0.96 (0.64–1.43)		0.83 (0.55–1.26)
p for heterogeneity[5]					0.57	
Dietary vitamin B6 (mg/d)						
≤ 1.55	203/205	0.98 (0.61–1.59)	91/90	1.08 (0.58–1.99)	92/100	0.84 (0.45–1.60)
1.56–1.95	210/205	1.16 (0.78–1.71)	97/91	1.27 (0.75–2.14)	101/99	1.15 (0.67–1.98)
1.96–2.40	232/207	1.24 (0.88–1.74)	121/104	1.42 (0.89–2.26)	94/86	1.19 (0.72–1.96)
≥ 2.40	177/205	1.00	79/103	1.00	87/89	1.00
p for trend[3]		0.80		0.95		0.53
Continuous after log2-transformation[4]		0.84 (0.56–1.26)		0.87 (0.53–1.42)		0.79 (0.47–1.34)
p for heterogeneity[5]					0.72	
Dietary vitamin B12 (µg/d)						
≤ 4.76	182/205	0.79 (0.53–1.16)	87/98	0.71 (0.42–1.21)	81/92	0.92 (0.53–1.59)
4.76–6.58	211/205	0.94 (0.67–1.33)	95/95	0.86 (0.53–1.40)	94/87	1.06 (0.65–1.74)
6.63–8.93	194/206	0.92 (0.67–1.27)	99/104	0.97 (0.62–1.51)	81/94	0.80 (0.50–1.28)
≥ 8.91	235/206	1.00	107/91	1.00	118/101	1.00
p for trend[3]		0.26		0.19		0.90
Continuous after log2-transformation[4]		0.84 (0.70–1.02)		0.80 (0.62–1.04)		0.85 (0.65–1.11)
p for heterogeneity[5]					0.73	

[1]The Total UCC group includes urothelial cell papillomas and carcinomas (morphology codes 8,120 and 8,130, and behaviour coded as uncertain whether benign or malignant, carcinoma *in situ*, and/or malignant) but excludes inverted papillomas (8,121/1). The Aggressive UCC category includes all stage T1 or higher, carcinoma *in situ*, or WHO 1973 grade 3 carcinomas (including Ta grade 3). The group Non-aggressive UCC category includes all stage Ta grade 1 or Ta grade 2 carcinomas.
[2]All values are odds ratios; 95% CIs in parentheses. Analyses were matched for age at blood collection, study centre, sex, date and time of blood collection, and fasting status and further adjusted for smoking status, duration and intensity of smoking, energy intake, red meat intake, processed meat intake, alcohol intake, physical activity, BMI, educational level.
[3]Test for trend was performed using the median log2 value of each quartile.
[4]Values were derived from adjusted models as described in footnote 2 for risk associated with a halving in dietary intake.
[5]Likelihood ratio test of heterogeneity, testing for a common association across subtypes.

tumours displayed a hypomethylated phenotype whereas invasive tumours showed widespread hypermethylation, confirming that the two pathways differ epigenetically.[28] Due to the relatively large number of cases in our study, we were able to analyse prognostic subgroups of UCC according to tumour aggressiveness. We found stronger associations for folate with aggressive UCC, which may have been missed in previous studies limited to total UCC. It is known that

aggressive, high-grade bladder tumours frequently show defects in the *TP53* and *RB* tumour suppressor genes.[29] In laboratory models, folate deficiency appears to induce *p53* mutations.[1] Moreover, even in the absence of genomic hypomethylation, folate depletion has been shown to induce hypomethylation in the coding region of *p53* while supplemental folate has been shown to revert the hypomethylation of this region.[1] This potential mechanism suggests that folate

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Table 4. Joint effects of smoking status and serum folate

	Smoking status						
	Never smokers		Former smokers		Current smokers		
	No. of cases/ controls	Total UCC[1,2]	No. of cases/ controls	Total UCC[1,2]	No. of cases/ controls	Total UCC[1,2]	*P*-interaction[3]
Serum folate (nmol/L)							
≤ 11.92	35/74	0.94 (0.52–1.71)	63/64	2.36 (1.34–4.17)	148/61	6.26 (3.62–10.81)	
11.95–16.02	46/76	1.33 (0.77–2.28)	77/67	2.83 (1.65–4.87)	82/56	3.33 (1.93–5.75)	
16.03–21.32	29/82	0.72 (0.41–1.28)	72/65	2.54 (1.48–4.37)	74/55	3.39 (1.94–5.90)	
≥ 21.33	42/77	1.00	75/80	2.05 (1.19–3.52)	58/44	2.88 (1.64–5.06)	0.07

[1]The Total UCC group includes urothelial cell papillomas and carcinomas (morphology codes 8,120 and 8,130, and behaviour coded as uncertain whether benign or malignant, carcinoma *in situ*, and/or malignant) but excludes inverted papillomas (8,121/1).
[2]All values are odds ratios; 95% CIs in parentheses. Analyses were matched for age at blood collection, study centre, sex, date and time of blood collection, and fasting status.
[3]Statistical interaction on a multiplicative scale was tested by introducing a product term between serum one-carbon metabolism biomarkers (quartiles) and smoking status in the model.

can modulate aggressive bladder carcinogenesis through DNA methylation. Within the EPIC cohort, associations for plasma carotenoids were particularly driven by aggressive UCC as well.[12] However, the precise mechanisms underlying the association with aggressive bladder cancer risk remain unresolved. Efficient one-carbon metabolism requires not only folate, but also vitamins B2, B6 and B12. These nutrients interact metabolically with folate in the one-carbon metabolism process, and may influence cancer risk.[1] Our study found, however, no evidence for an association of blood concentrations and dietary intake of vitamins B2, B6 and B12 with UCC risk.

Smoking may affect B-vitamin status and reduce bioavailability of folate. Current smokers have lower circulating folate concentrations, even after correction for folate intake. This suggests that smoking alters the systemic uptake or metabolism of folate.[23,24] Previous findings suggest that the combined effects of smoking with decreased folate levels can induce increased chromosomal damage. The higher proportion of cells with chromosome aberrations in cigarette smokers was attributed to lower folate levels in smokers compared to non-smokers.[30,31] Thus, DNA damage induced by smoking may be modulated by the folate metabolic pathway. In the present study, a statistically significant increased risk of UCC was only observed in current smokers with lowest folate concentrations. Consistent results were seen in a case–control study from the United States where current smokers with lower folate intake tended to have a greater risk of bladder cancer compared to current smokers with higher folate intake.[32] If an effect of serum folate exists, our study suggests that it is particularly prominent in smokers with lowest folate concentrations. No statistical interaction with alcohol intake, gender, and BMI was observed. However, the observed associations should be interpreted with caution because we conducted multiple comparisons and because residual confounding by smoking cannot be excluded.

Diets with a low intake of fruits and vegetables, leading to insufficient folate intake and serum levels, may reflect an unhealthy lifestyle.[33] In our study, participants with lower serum folate concentrations tended to have an unhealthier lifestyle. However, this is unlikely to explain the observed associations of serum folate with UCC risk, since risk estimates remained unchanged after adjustment for several lifestyle factors. On the other hand, the observed associations may reflect other unmeasured lifestyle factors associated with both low serum folate concentrations and high risk of UCC. Further, exposure to potential risk factors may have changed during follow-up, leading to residual confounding. We could not take this into account, as replicate measurements were not available. Higher folate concentrations may also be a marker of a concerted effect of multiple bioactive compounds in fruit and vegetables. Although folate sources are not restricted to fruit and vegetables, blood folate concentrations may be a useful biomarker for the intake of fruit and vegetables in populations consuming unfortified foods.[34] At the time of data collection, no European country had introduced mandatory folic acid fortification, although voluntary fortification was accepted in some countries, including France and the UK.[35] The use of dietary supplements could also affect serum concentrations of micronutrients. However, a study on the use of dietary supplements in a sub-sample of the EPIC cohort indicated that folic acid supplements were not frequently consumed at the time of data collection.[36] Also, no substantial change in risk estimates was observed by simple adjustment for dietary supplement use (yes *vs.* no) (data not shown). Therefore, serum folate in our study seems to be largely determined by natural folates from foods. Despite the low correlation between serum concentrations and dietary intakes of folate (0.22), we observed quite similar risk estimates in the smoking adjusted but not the fully adjusted models. The weak correlation may be explained by errors in the food composition table from which folate was derived and biases in the assessment of dietary intake. Other explanations may be related to the bioavailability of folate that is influenced by several factors including smoking.

The strengths of our study include its prospective design, a relatively large number of incident UCC cases, the wide range in blood concentrations of one-carbon metabolism related nutrients, and the collection of blood samples before cancer diagnosis. However, some limitations of the present study need to be discussed. Our analyses were based on single measurements of nutrients involved in the one-carbon metabolism at baseline, assuming stable serum concentrations during follow-up. Blood concentrations may differ over time because of day-to-day variation and long-term changes within persons, leading to an attenuation of the associations. A reliability study in a subsample of the two Dutch cohorts within EPIC showed that over 2–5 years, serum homocysteine and vitamin B12 were highly reliable biomarkers with intraclass correlation coefficients (ICC) of 0.91 and 0.75, respectively.[37] The ICC for serum folate was 0.45 and for vitamin B6 was 0.38. These measures should be reliable enough to detect moderate associations. Serum folate is an indicator of more recent folate intake, while red blood cell folate is considered to reflect average concentration over the erythrocyte life span (about 120 days).[38,39] As red blood cells are less subject to short-term dietary changes and reflect long-term folate status, the observed associations for serum folate may be even stronger for red blood cell folate. We did not have information on polymorphisms in *MTHFR* or other relevant genes, which play an important role in DNA methylation and are determinants of serum folate concentrations.[40] Possible statistical interaction between serum folate concentrations and polymorphisms in *MTHFR* in relation to UCC risk needs to be investigated.

In conclusion, our study suggests that lower serum folate concentrations may increase the risk of UCC, in particular aggressive UCC. This increased risk was only seen in current smokers. However, residual confounding by smoking cannot be excluded and confirmation in prospective studies with multiple measurements is warranted.

Authors' contributions
The authors' responsibilities were as follows - AV, HBB-D-M, MMR, EK, KKA, FLB, EHJ, and LAK: statistical analyses and drafting of the manuscript; HBB-D-M: recruitment and follow-up of the Bilthoven cohort; NR and ATjønneland: recruitment and follow-up of the Aarhus and Copenhagen cohort; MCB-R, CC: recruitment and follow-up of the French cohort; JC-C and RK: recruitment and follow-up of the Heidelberg cohort; SW and HB: recruitment and follow-up of the Potsdam cohort; A Trichopoulou, PL, and DT: recruitment and follow-up of the Greek cohort; SS, DP, and SP: recruitment and follow-up of the 5 Italian cohorts; PHP: recruitment and follow-up of the Utrecht cohort; EW and GS: recruitment and follow-up of the Norway cohort; PJ, M-DC, EA, M-JS: recruitment and follow-up of the 5 Spanish cohorts; RE and JM: recruitment and follow-up of the Malmö cohort; BL: recruitment and follow-up of the Umeå cohort; K-TK and NW: recruitment and follow-up of the Cambridge and Oxford cohort; and PB and ER: coordination of the entire EPIC collaboration. All authors contributed to the interpretation of the results and preparation and approval of the final manuscript. None of the authors had a conflict of interest.

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