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A prospective study of one-carbon metabolism biomarkers and cancer of the head and neck and esophagus

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Abbreviations: CI: confidence interval; CRC: colorectal cancer; CV: coefficient of variations; EPIC: European Prospective Investigation into Cancer and Nutrition; ESCC: esophagus squamous cell carcinoma; HNC: head and neck cancer; HR: hazard ratio; OCM: one-carbon metabolism; OR: odds ratio; SCC: squamous cell carcinoma

Additional Supporting Information may be found in the online version of this article.

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Experimental and epidemiological data suggest that factors of one-carbon metabolism are important in the pathogenesis of several cancers, but prospective data on head and neck cancer (HNC) and esophagus cancer are limited. The European Prospective Investigation into Cancer and Nutrition (EPIC) study recruited 385,747 participants from 10 countries who donated a blood sample. The current study included 516 cancer cases of the head and neck and esophagus and 516 individually matched controls. Plasma levels of vitamins B2, B6, B9 (folate), B12, and methionine and homocysteine were measured in pre-diagnostic plasma samples and analyzed in relation to HNC and esophagus cancer risk, as well as post-diagnosis all-cause mortality. After controlling for risk factors, study participants with higher levels of homocysteine had elevated risk of HNC, the odds ratio (OR) in conditional analysis when comparing the top and bottom quartiles of homocysteine [$OR_{Q4\ vs.\ Q1}$] being 2.13 (95% confidence interval [95% CI] 1.13–4.00, *p* for trend 0.009). A slight decrease in HNC risk was also seen among subjects with higher levels of folate ($OR_{Q4\ vs.\ Q1}$ 0.63, 95% CI 0.35–1.16, *p* for trend 0.02). Subgroup analyses by anatomical sub-site indicated particularly strong associations with circulating homocysteine for oral cavity and gum cancer (*p* for trend 8×10^{-4}), as well as for oropharynx cancer (*p* for trend 0.008). Plasma concentrations of the other investigated biomarkers did not display any clear association with risk or survival. In conclusion, study participants with elevated circulating levels of homocysteine had increased risk of developing squamous cell carcinoma of the head and neck.

What's new?

One-carbon metabolism (OCM) involves the transfer of a carbon unit from methyl donor nutrients to molecules involved in the synthesis and methylation of DNA. As a result, dietary imbalances or deficiencies in nutrients crucial for OCM may affect DNA replication, repair, and regulation, potentially facilitating cancer development. This analysis of circulating levels of OCM nutrients in head and neck cancer and esophageal cancer patients and matched controls reveals an association between elevated levels of the amino acid homocysteine and increased risk of squamous cell carcinoma of the head and neck. Risk was decreased slightly by elevated folate levels.

In 1981 Doll and Peto estimated that approximately 35% of cancer deaths in the United States could be avoided by modification of diet.[1] While little evidence has subsequently emerged on food groups linked to specific cancer sites, cancers of the head and neck (HNC) consistently occur less frequently among subjects with a high consumption of fruits and vegetables, with supporting data from both case–control[2] and prospective studies.[3,4]

Fruits and vegetables are important dietary sources of some B-vitamins and additional nutrients that are involved in the one-carbon metabolism (OCM).[5–7] The metabolic pathway of OCM has been frequently implicated in carcinogenesis because of its involvement in maintaining nucleotide synthesis and methylation. Imbalances and deficiencies among crucial OCM nutrients may interfere with DNA replication, DNA repair and regulation of gene expression, any of which could promote carcinogenesis.[8,9]

The one-carbon metabolism pathway has been frequently investigated in relation to multiple cancer types in prospective studies,[10–14] but not yet in relation to head and neck cancer. Recently, Johansson *et al.* reported that elevated serum levels of both vitamin B6 and methionine were associated with a reduced risk of lung cancer of approximately 50%, independently of smoking status.[12] These results, as well as similar studies on other cancer sites such as colorectal cancer,[15] suggest that the one-carbon metabolism biomarkers may provide important information on disease risk for some specific cancers.

In order to obtain evidence on the importance of one-carbon metabolism in HNC and esophagus cancer, we

conducted a large nested case–control study within the European Prospective Investigation into Cancer and Nutrition (EPIC).

Material and Methods

Study cohort

The study was nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) study. EPIC recruitment procedures, collection of questionnaire data, anthropometric measurements, and blood samples have been described in detail.[16] In brief, 521,330 individuals were recruited to the cohort between 1992 and 2000 from 10 European countries, of whom 385,747 contributed a blood sample. Blood fractions were aliquoted into 0.5 mL straws, which were heat-sealed and stored in liquid nitrogen tanks at −196°C, except in Umeå, Sweden, where samples were stored in 1.8 mL plastic tubes in −80°C freezers. Participants completed self-administered questionnaires on lifestyle factors and diet.

Follow-up for cancer incidence

Incident cancer cases were identified at regular intervals through population-based cancer registries (Denmark, Italy except Naples, the Netherlands, Norway, Spain, Sweden and United Kingdom) or by active follow-up (France, Germany, Greece and Naples), which involved a combination of methods, including review of health insurance records, cancer and pathology registries, as well as direct contact with participants and their next-of-kin.

Mortality data, including vital status, cause of death, and date of death, were obtained from mortality registries at the regional or national level. Subjects were followed up from study entry until cancer diagnosis (except non-melanoma skin cancer), death, emigration, or the end of the follow-up period for the relevant study centre. End of follow-up was defined as the latest date of complete follow-up for both cancer incidence and vital status and varied between study centres from December 2004 to June 2010. Vital status at follow-up is over 98% complete.

Selection of case and control participants

We initially identified 1,273 subjects diagnosed with incident head and neck or esophagus cancer within the entire EPIC cohort by the end of the follow-up period for all centres. These cancer cases were defined on the basis of the *International Classification of Diseases for Oncology, Second Edition* (ICD-O-2), and included: oral cavity (ICD C02.0-C02.9, C04.0-C04.9, C03.0-C03.9, C05.0-C06.9, C14.0-C14.9), oropharynx (C01.9, C02.4, C09.0-C10.9), hypopharynx (C13.0-C13.9), larynx (C32.0-C32.9), and esophagus (C15.0-C15.9). Cases who did not donate a blood sample ($n = 152$), did not have enough plasma available for biochemical analysis ($n = 20$), had a history of another cancer ($n = 158$, except non melanoma skin cancer), were not histologically confirmed, were prevalent at the time of blood donation, or did not have questionnaire information available ($n = 22$), were excluded, leaving 921 eligible cases. Because the etiology of squamous cell carcinoma (SCC) and adenocarcinoma are thought differ and the vast majority of HNC are SCC, we excluded adenocarcinoma of the head and neck ($n = 16$). However, because esophagus cancers are more evenly split between adenocarcinoma and SCC, we retained all esophagus cancer cases, but focus primarily on the SCC in our analysis. After excluding cases from Denmark ($n = 288$) and the Malmö centre in Sweden ($n = 101$) who did not participate in this study, 516 eligible case participants with plasma samples were available for biochemical analyses. Data on histology were collected from each centre where possible.

For each case participant, one control was randomly chosen from appropriate risk sets consisting of all cohort members alive and free of cancer (except non-melanoma skin cancer) at the time (and hence age) 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). In addition, we included 479 additional controls (control group 2) that were analysed in the context of a parallel study and individually matched to cases of another cancer site using identical matching criteria.

The final dataset included 516 cancer cases and 516 individually matched controls, as well as 479 additional unmatched controls from control group 2 that contributed to unconditional and stratified risk analyses.

Biochemical analyses

All biochemical analyses were performed at Bevital A/S (http://www.bevital.no), Bergen, Norway. The study included measurements of plasma levels of B2 (riboflavin), B6 (measured as pyridoxal 5'-phosphate, its active form), folate (B9), B12 (cobalamin), total homocysteine, and methionine. All case and control participants were successfully analyzed, for at least one of the biomarker. We also measured cotinine as an indicator of recent smoking behavior. Levels of B2, B6, 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)[17,18] and microbiological methods were used to determine levels of folate (*Lactobacillus casei*)[19,20] and B12 (*Lactobacillus leichmannii*).[21]

Samples were analysed in batches of 86 and quality control included six calibration samples, two control samples, and one blank sample in each batch. The coefficients of variations (CVs) within and between batches were, respectively, 6 and 11% for vitamin B2, 3% and 6% for vitamin B6, 4% and 5% for folate and vitamin B12, 1% and 3% for methionine, and 1% and 2% for homocysteine. All plasma samples were kept at −80°C and all HNC and esophagus cancer cases and their individual matched controls were analyzed together within the same batches in random order, as were samples

from control group 2. The laboratory staff was blinded to the case–control status of the blood samples.

Statistical analyses

The relation of lifestyle and dietary factors with biomarker levels were assessed using linear regression models, adjusted for age, sex, and country.

Risk analysis involved calculating quartiles of plasma levels for each biomarker of interest based on their distribution among the matched control participants. Odds ratios (OR) and 95% confidence intervals (CI) for participants in the second, third, and fourth quartiles were calculated relative to the first quartile using conditional logistic regression, conditioning on individual case sets. Additional adjustment was conducted for indicators of risk factors, including smoking status [never, former, current, missing] and for quartiles of cotinine levels [defined by the distribution among current smokers], which was considered to be the most accurate measure of smoking intensity at the time of blood collection, as well as educational attainment (in four categories) and alcohol consumption at the time of recruitment (g/day). Adjusting for additional smoking variables (duration of smoking, average cigarettes smoked per day) did not alter the results notably and were not included in the final models.

In order to increase the statistical power and further evaluate the consistency of any association observed in the conditional logistic regression, we conducted a risk analysis including the 479 additional controls that were available using unconditional logistic regression adjusted for matching factors, including age at recruitment, sex, country, as well as risk factors using the same approach as in the conditional logistic regression outlined above.

Stratified analysis by tumor site was also conducted using unconditional logistic regression adjusting for matching factors (age at recruitment, sex, country), as well as risk factors (educational attainment, cotinine, smoking status and alcohol consumption at the time of recruitment).

As a measure of statistical significance for each biomarker and statistical model, we included the base 2 logarithm ($\log_2$) of the biomarker levels as a continuous variable in a separate logistic regression model to estimate the p for trend. This model was also used in exploratory stratified risk analysis by pre-defined demographic characteristics and risk factors, and the OR trend estimate from this model ($\log_2$OR) may be interpreted as the relative risk associated with a doubling in plasma levels of the biomarker of interest. We used χ^2 tests to assess heterogeneity in $\log_2$OR estimates in stratified analyses.

All-cause mortality for differences in biomarker levels was evaluated among HNC and esophagus squamous cell carcinoma (ESCC) cases by Cox proportional hazard regression analyses using years since diagnosis as the time variable. Hazard ratios (HR) of all-cause mortality were calculated after adjusting for age at diagnosis, sex and country.

All p values were two-sided and statistical analyses were conducted using SAS version 9.2 (Cary, NC).

Results

Baseline characteristics

The final study population included 350 head and neck cancers and 166 esophagus cancers, and 516 individually matched controls. 68% of the nested case–control population were male and 32% were female (Table 1). The median age at recruitment was 62 years (5th–95th percentile: 49–77) and the average time from blood draw to diagnosis for cases was 6.4 years. Control group 2 included an additional 479 subjects with similar demographic characteristics as the matched control group, but with a higher proportion of women.

Correlates of one-carbon metabolism biomarkers

The relation of dietary intake of major food groups, smoking, and alcohol intake with biomarker levels are presented in Supporting Information Table 1. Dietary intake of vegetable, of dairy products and meat products were inversely associated with homocysteine. Current smokers also had a notably lower levels of riboflavin ($p < 10^{-6}$), vitamin B6 ($p < 10^{-3}$) and folate ($p < 10^{-3}$), than did never smokers. Additionally, correlation coefficients between the measured biomarkers are presented in Supporting Information Table 2.

Plasma one-carbon metabolism biomarkers in relation to HNC and esophagus cancer risk

We conducted the main risk analysis for HNC and ESCC separately (Table 2). After adjusting for education, alcohol consumption, smoking status and cotinine, study participants with higher levels of homocysteine had an elevated risk of HNC, the OR when comparing the top and bottom quartiles [$OR_{Q4\ vs.\ Q1}$] being 2.13 (95% confidence interval [95% CI], 1.13–4.00, p for trend 0.009). A decrease in risk was also seen among subjects with higher levels of folate ($OR_{Q4\ vs.\ Q1}$ 0.63, 95% CI 0.35-1.16, p for trend 0.02). Plasma levels of the different biomarkers displayed weak or no evidence of association with ESCC risk (p for trend >0.06). Including the additional controls in an unconditional logistic regression did not notably affect the association between homocysteine and HNC risk (p for trend 0.002), but the association of folate was attenuated and no longer statistically significant (p for trend 0.39). When comparing HNC cases with control group 2 only, homocysteine displayed similar associations as in the conditional analysis ($OR_{Q4\ vs.\ Q1}$ 2.30, 95% CI 1.47–3.59, P for trend 3×10^{-4}, and $OR_{Q4\ vs.\ Q1}$ 1.70, 95% CI 1.06–2.78, p for trend 0.03 for the minimally adjusted and fully adjusted model, respectively).

Stratified risk analysis by tumor site (Table 3) indicated that the association of homocysteine was particularly prominent for cancers of the oral cavity and gum (P for trend 8×10^{-4}) and oropharynx cancer (p for trend 0.008), whilst no association was seen for ESCC, larynx and hypopharynx cancer or for adenocarcinoma of the esophagus (p for trend >0.09).

Overall, a doubling in plasma homocysteine was associated with a 53% higher odds of HNC and ESCC combined

Table 1. Baseline and clinical characteristics of study participants

	No. (%) participants in group		
	Cases (n = 516)	Matched controls (n = 516)	Control group n 2 (n = 479)
Discrete variables			
Participating countries			
France	7 (1)	7 (1)	13 (3)
Italy	65 (13)	65 (13)	93 (19)
Spain	93 (18)	93 (18)	59 (12)
United Kingdom	126 (24)	126 (24)	71 (15)
The Netherlands	71 (14)	71 (14)	52 (11)
Greece	21 (4)	21 (4)	18 (4)
Germany	98 (19)	98 (19)	131 (27)
Sweden	34 (7)	34 (7)	37 (8)
Norway	1 (0)	1 (0)	5 (1)
Sex			
Men	353 (68)	353 (68)	255 (53)
Women	163 (32)	163 (32)	224 (47)
Smoking status			
Never	105 (20)	214 (41)	220 (46)
Former	145 (28)	184 (36)	160 (33)
Years since quitting <10	55 (40)	47 (26)	44 (28)
Years since quitting ≥10	84 (60)	131 (74)	113 (72)
Current	256 (50)	104 (20)	96 (20)
Unknown	10 (2)	14 (3)	3 (1)
Education			
None	36 (7)	31 (6)	33 (7)
Primary school	191 (38)	170 (34)	158 (33)
Technical/professional school	114 (22)	136 (27)	104 (22)
Secondary school	78 (15)	64 (13)	64 (13)
Higher education	72 (14)	95 (19)	105 (22)
Unknown	18 (4)	9 (2)	13 (3)
Alcohol intake at recruitment (g/d)			
=0	89 (17)	60 (12)	57 (12)
0.1–6	136 (26)	167 (32)	175 (37)
6.1–12	48 (9)	77 (15)	58 (12)
12.1–24	71 (14)	92 (18)	90 (19)
24.1–60	109 (21)	94 (18)	81 (17)
60.1–96 in men or >60 in women	46 (9)	19 (4)	15 (3)
>96 in men	15 (3)	7 (1)	3 (1)
Body mass index[1]			
<18.5	9 (2)	5 (1)	1 (0)
18.5–25	192 (37)	201 (39)	190 (40)
25–30	230 (45)	239 (46)	207 (43)
≥30	85 (16)	71 (14)	81 (17)
Continuous variables, median (5th-95th percentile)			
Age at blood draw (yrs)	57 (42–71)	57 (42–71)	57 (41–68)

Table 1. Baseline and clinical characteristics of study participants (Continued)

	No. (%) participants in group		
	Cases (n = 516)	Matched controls (n = 516)	Control group n 2 (n = 479)
Serum levels for components of the 1-carbon metabolism			
Riboflavin, nmol/L	13.4 (5.3–49.9)	13.1 (5.9–47.9)	13.8 (5.4–44.4)
Pyridoxal phosphate, nmol/L	33.1 (13.9–101)	34.5 (14.2–98.8)	34.1 (14.2–89.2)
Serum folate, nmol/L	12.5 (3.5–41.3)	12.9 (5.1–36.4)	12.1 (4.3–38.5)
Cobalamin, Vit B12, pmol/L	319 (173–577)	328 (176–521)	337 (186–588)
Homocysteine, μmol/L	10.8 (6.7–23.9)	10.2 (6.5–18)	9.8 (6.0–17.7)
Methionine, μmol/L	25.1 (16.6–37.2)	25.1 (17.2–37.9)	24.6 (16.8–35.1)
Clinical characteristics, case participants only			
Age at diagnosis, median (range), yrs	62 (49–77)		
Time from blood draw to diagnosis	6.4 (0.75–12.8)		
Tumour site, no. (%)			
Esophagus	166 (32)		
Hypopharynx + larynx	145 (28)		
Gum + oral cavity	110 (21)		
Oropharynx	67 (13)		
Other	28 (6)		

[1]Body mass index is calculated as weight in kilograms divided by height in meters squared.

(OR for $\log_2$ homocysteine [OR_{log2}]; 1.53, 95% CI: 1.17–1.98, p for trend 0.001). The relation between homocysteine and squamous cell HNC/ESCC cancer by demographic characteristics and risk factors did not display any clear effect modifications in stratified analysis (p for heterogeneity >0.21) (Fig. 1 and Supporting Information Fig. 1). Notably, the inverse association of homocysteine with risk was observed both among never smokers (OR_{log2} 1.88, 95% CI 1.08–3.28), as well as among current smokers (OR_{log2} 1.56, 95% CI 1.03–2.35). Stratified risk analyses were also conducted for vitamin B2 (Supporting Information Fig. 2), B6 (Supporting Information Fig. 3), folate (Supporting Information Fig. 4), B12 (Supporting Information Fig. 5) and methionine (Supporting Information Fig. 6).

All-cause mortality for study participants diagnosed with HNC and esophagus cancer

Results of cox-proportional hazards regression models of all-cause mortality based on 277 deaths are shown in Supporting Information Table 3. Overall, plasma levels of the investigated OCM biomarkers did not display strong associations with all-cause mortality, nor with HNC-specific cause mortality (94 deaths). However, both cobalamin and methionine were weakly associated with all-cause mortality among HNCs in the unadjusted analysis, the hazard ratio (HR) when comparing the top and bottom quartiles [$HR_{Q4\ vs.\ Q1}$] being 0.60 (95% CI: 0.38–0.96, p for trend 0.06) and 0.69 (95% CI: 0.44–1.11, p for trend 0.01), respectively, and adjusting for potential confounders did not notably affect the HR estimates (Supporting Information Table 3).

Discussion

This is the first study investigating pre-diagnostic biomarkers of the one-carbon metabolism in relation to cancers of the head and neck and esophagus. We measured plasma levels of several B-vitamins and related metabolites, and observed that subjects with elevated levels of homocysteine and low levels of folate had higher risk of HNC. The other investigated biomarkers, including methionine and vitamins B2, B6, folate and B12, were not associated with risk of HNC or ESCC.

Homocysteine and cancers of the head and neck and esophagus

Only a few retrospective case–control studies have reported on circulating OCM biomarkers and HNC risk, most indicating lower folate levels and higher homocysteine levels among cases than among controls.[22–25] However, given the potential for reverse causality to influence the results in retrospective case–control studies, it is difficult to interpret those studies in the context of cancer etiology. We are not aware of any well-powered prospective studies investigating circulating one-carbon metabolism biomarkers and HNC cancer. This is noteworthy considering that head and neck cancers have been widely linked to dietary intake of fruits and vegetables that are important sources for specific B-vitamins involved in

Table 2. Odds ratios and 95% confidence intervals of head and neck cancer (HNC) and esophagus squamous cell carcinoma (ESCC) for plasma levels of vitamins B2, B6, folate, B12, homocysteine and methionine

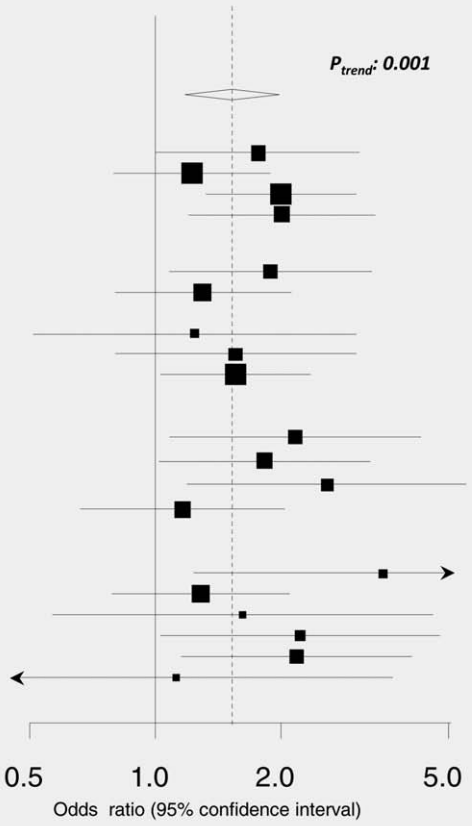
			Odds ratio (95% confidence interval)					
			Head and neck cancer[1]			Esophagus squamous cell carcinoma		
Quartile (Range)	Case[2]	Control[2]	Matched controls unadjusted[3] (n = 350/350)[3]	Matched controls adjusted[4] (n = 350/350)[4]	All controls combined adjusted[5] n = (350/995)[5]	Matched controls unadjusted[6] (n = 73/73)[6]	Matched controls adjusted[4] (n = 73/73)[4]	All controls combined adjusted[7] (n = 73/995)[7]
Riboflavin (Vitamin B2), nmol/L[8]								
1 (2.5–9.4)	152 (29.5%)	129 (25.0%)	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]
2 (9.5–13.1)	96 (18.6%)	129 (25.0%)	0.61 (0.40–0.94)	0.75 (0.45–1.27)	0.76 (0.51–1.13)	0.47 (0.16–1.36)	0.17 (0.02–1.26)	0.50 (0.21–1.19)
3 (13.2–21.3)	145 (28.1%)	129 (25.0%)	1.01 (0.66–1.52)	1.33 (0.80–2.20)	1.12 (0.77–1.64)	0.66 (0.25–1.79)	0.57 (0.09–3.56)	1.05 (0.50–2.20)
4 (21.4–199)	123 (23.8%)	129 (25.0%)	0.67 (0.42–1.05)	1.19 (0.68–2.08)	1.18 (0.77–1.79)	0.66 (0.24–1.79)	0.94 (0.18–4.92)	1.21 (0.54–2.72)
p for trend[9]			0.21	0.22	0.31	0.33	0.84	0.41
Pyridoxal 5'-phosphate (Vitamin B6), nmol/L[8]								
1 (7.2–25.6)	160 (31%)	129 (25.0%)	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]
2 (25.7–34.5)	116 (22.5%)	129 (25.0%)	0.67 (0.44–1.03)	0.75 (0.45–1.26)	0.92 (0.63–1.34)	0.88 (0.34–2.27)	1.96 (0.51–7.56)	1.09 (0.49–2.42)
3 (34.6– 47.6)	112 (21.7%)	129 (25.0%)	0.66 (0.43–1.02)	0.96 (0.57–1.63)	1.09 (0.73–1.61)	1.05 (0.38–2.92)	1.41 (0.28–7.18)	1.34 (0.59–3.04)
4 (47.7–272)	128 (24.8%)	129 (25.0%)	0.77 (0.50–1.17)	1.01 (0.59–1.71)	1.02 (0.69–1.51)	1.31 (0.52–3.27)	1.82 (0.45–7.42)	2.26 (1.06–4.84)
p for trend[9]			0.41	0.48	0.56	0.69	0.82	0.06
Serum folate (vitamin B9), nmol/L[8]								
1 (0.3–9.1)	163 (31.6%)	129 (25.0%)	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]
2 (9.2–12.8)	108 (20.9%)	129 (25.0%)	0.51 (0.33–0.81)	0.62 (0.36–1.08)	0.80 (0.54–1.17)	1.08 (0.39–2.98)	7.78 (1.24–49.05)	1.04 (0.48–2.22)
3 (12.9– 18.1)	119 (23.1%)	129 (25.0%)	0.61 (0.39–0.98)	0.77 (0.44–1.36)	1.13 (0.77–1.67)	0.85 (0.31–2.36)	4.08 (0.74–22.60)	1.11 (0.49–2.49)
4 (18.2–109)	126 (24.4%)	129 (25.0%)	0.48 (0.30–0.80)	0.62 (0.34–1.13)	0.92 (0.61–1.38)	1.18 (0.38–3.68)	12.45 (1.14–135.5)	1.03 (0.47–2.24)
p for trend[9]			2×10^{-4}	0.02	0.39	0.44	0.14	0.69
Cobalamin (Vitamin B12), pmol/L[8]								
1 (75.1–265)	148 (28.7%)	129 (25.0%)	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]
2 (266–328)	131 (25.4%)	129 (25.0%)	0.83 (0.54–1.27)	1.01 (0.61–1.68)	0.88 (0.61–1.29)	1.33 (0.43–4.10)	1.49 (0.33–6.73)	1.06 (0.50–2.24)
3 (329– 391)	92 (17.8%)	129 (25.0%)	0.58 (0.36–0.92)	0.59 (0.33–1.05)	0.66 (0.44–0.99)	0.75 (0.25–2.27)	1.07 (0.27–4.32)	0.81 (0.37–1.80)
4 (392–2737)	145 (28.1%)	129 (25.0%)	0.91 (0.58–1.44)	1.20 (0.68–2.10)	0.90 (0.62–1.30)	1.10 (0.34–3.55)	0.94 (0.21–4.25)	1.07 (0.51–2.23)
p for trend[9]			0.88	0.64	0.63	0.85	0.78	0.98
Homocysteine, μmol/L[8]								
1 (4.9–8.3)	109 (21.1%)	128 (24.8%)	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]
2 (8.4- 10.1)	123 (23.8%)	130 (25.2%)	1.35 (0.85–2.13)	1.28 (0.74–2.21)	1.26 (0.85–1.89)	0.80 (0.29–2.24)	0.52 (0.12–2.24)	1.07 (0.48–2.36)

Table 2. Odds ratios and 95% confidence intervals of head and neck cancer (HNC) and esophagus squamous cell carcinoma (ESCC) for plasma levels of vitamins B2, B6, folate, B12, homocysteine and methionine (Continued)

			Odds ratio (95% confidence interval)					
			Head and neck cancer[1]			Esophagus squamous cell carcinoma		
Quartile (Range)	Case[2]	Control[2]	Matched controls unadjusted[3] (n = 350/350)[3]	Matched controls adjusted[4] (n = 350/350)[4]	All controls combined adjusted[5] n = (350/995)[5]	Matched controls unadjusted[6] (n = 73/73)[6]	Matched controls adjusted[4] (n = 73/73)[4]	All controls combined adjusted[7] (n = 73/995)[7]
3 (10.2– 12.4)	96 (18.6%)	129 (25%)	1.12 (0.70–1.79)	1.01 (0.58–1.76)	0.88 (0.58–1.36)	1.05 (0.37–3.00)	0.67 (0.16–2.71)	0.74 (0.31–1.74)
4 (12.5–64.9)	188 (36.4%)	129 (25%)	2.81 (1.66–4.75)	2.08 (1.11–3.90)	1.81 (1.19–2.74)	1.67 (0.63–4.43)	1.09 (0.27–4.32)	1.69 (0.76–3.74)
p for trend[9]			5×10^{-5}	0.009	0.002	0.07	0.52	0.11
Methionine, μmol/L[8]								
1 (12.6–21.8)	149 (28.9%)	129 (25.0%)	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]
2 (21.9–25.1)	110 (21.3%)	129 (25.0%)	0.72 (0.48–1.07)	0.91 (0.57–1.45)	0.83 (0.57–1.22)	0.78 (0.31–1.94)	1.59 (0.25–10.01)	0.76 (0.36–1.61)
3 (25.2– 29.3)	131 (25.4%)	129 (25.0%)	0.86 (0.56–1.32)	1.21 (0.71–2.06)	0.94 (0.64–1.38)	0.60 (0.22–1.64)	0.54 (0.10–3.00)	0.95 (0.44–2.04)
4 (29.4–62.9)	126 (24.4%)	129 (25.0%)	0.85 (0.54–1.34)	1.30 (0.73–2.31)	0.99 (0.67–1.48)	0.74 (0.25–2.19)	1.23 (0.20–7.69)	1.29 (0.62–2.68)
p for trend[9]			0.25	0.54	0.67	0.24	0.68	0.73

[1]Adenocarcinoma excluded. [2]Numbers include all cancer cases of the head and neck and esophagus cancer cases, and individually matched control for whom laboratory measurements were available for complete case sets. Uninformative case-sets were excluded. [3]Assessed by analysing HNC cases and their individually matched controls by conditional logistic regression, conditioning on individual case set. [4]Further adjusted for educational attainment (in four groups), smoking status (never/former/current/missing), cotinine (quartiles defined among current smokers) and alcohol intake at recruitment (g/d). [5]Assessed by analysing HNC cases and all controls combined by unconditional logistic regression, adjusting for country, sex, age at recruitment (in 5-year groups), educational attainment (in four groups), smoking status (never/former/current/missing), cotinine (quartiles defined among current smokers) and alcohol intake at recruitment (g/day). [6]Assessed by analysing ESCC cases and their individually matched controls by conditional logistic regression, conditioning on individual case set. [7]Assessed by analysing ESCC cases and all controls combined by unconditional logistic regression, adjusting for country, sex, age at recruitment (in 5-year groups), educational attainment (in four groups), smoking status (never/former/current/missing), cotinine (quartiles defined among current smokers) and alcohol intake at recruitment (g/d). [8]Quartile cut-off points were determined based on the plasma level distribution of each biomarker among the 516 individually matched controls of the head and neck and esophagus. [9]p for trend assessed by the base 2 logarithm of the serum levels.

study group	Cases[a]	Controls[a]	OR[b]	95%CI
Overall log2 - Homocysteine	443	994	1.53	1.17- 1.98
By site (p-heterogeneity= 0.51)[c]				
Esophagus squamous cell carcinoma	73	994	1.76	1.00- 3.09
Hypopharynx and Larynx	145	994	1.22	0.79- 1.88
Gum and Oral cavity	110	994	2.00	1.32- 3.02
Oropharynx	67	994	2.00	1.19- 3.37
By smoking status (p-heterogeneity= 0.74)[c]				
Never smokers	95	436	1.88	1.08- 3.28
Former smokers	110	341	1.30	0.80- 2.11
By time since quitting smoking (years)				
< 10	45	91	1.24	0.51- 3.00
>= 10	59	242	1.55	0.80- 3.01
Current smokers	233	200	1.56	1.03- 2.35
By time from blood draw to diagnosis (p-heterogeneity= 0.33)[c]				
1-35 months	111	227	2.01	1.10-3.70
36-75 months	115	251	1.73	1.04-2.89
76-107 months	104	239	2.35	1.20-4.61
108-151' months	108	267	1.17	0.72-1.91
By alcohol intake at recruitment (p-heterogeneity= 0.38)[c]				
=0 g/day	78	117	3.51	1.23-10.00
0.1-6 g/day	107	343	1.28	0.78- 2.10
6.1-12 g/day	40	134	1.61	0.57- 4.59
12.1-24 g/day	65	182	2.22	1.03- 4.78
24.1-60 g/day	96	174	2.18	1.16- 4.10
60.1-96 g/day	42	34	1.12	0.34- 3.70



[a] Head and neck and esophagus cancer cases and controls included in each stratified analysis (control group 2 was included).
[b] ORs were assessed by unconditional logistic regression by including the base 2 logarithm of plasma biomarker levels (ORs indicate relative risks of a doubling in plasma levels), and where relevant adjusted for age, sex, and country; the black dots indicate the ORs and the horizontal lines indicate the 95% confidence intervals.
[c] P heterogeneity indicates results of chi-square test assessing the null hypothesis of ORs being the identical.

Figure 1. Forest plot showing stratified OR of cancer of the head and neck and esophagus for log2-of plasma homocysteine.

the pathways of one-carbon metabolism.[2–4] In the current EPIC study we observed a higher risk for HNC among subjects with elevated plasma levels of homocysteine. Most prospective studies linking specific cancer sites to OCM have implicated other factors with risk, in particular vitamin B6,[12,15,26] rather than homocysteine. Indeed, a clear decrease in risk of subsequent lung cancer with elevated levels of vitamin B6 and methionine was observed in a recent lung cancer study within EPIC,[12] whereas no associations of those biomarkers were observed with risk in the current study on HNC and ESCC. One observation that could explain this contrasting result is that the findings for B6 and methionine with lung cancer were particularly relevant for small cell carcinoma and adenocarcinoma, while no clear association was found for squamous cell carcinoma, the histology representing the majority of cancer cases of the head and neck and esophagus.

In the EPIC study population, the increase in HNC risk was primarily observed among subjects in the top quartile of plasma homocysteine who experienced approximately double the risk of those in quartiles 1 to 3 (Table 2). Apart from larynx/hypopharynx cancer, this association was evident for all squamous cell cancers, including cancers of the esophagus, oral cavity and oropharynx, but not for adenocarcinoma of the esophagus (Table 3). When adjusting for potential confounders, including tobacco exposure and alcohol intake, the OR estimates were moderately attenuated (Table 2), but in stratified analysis the association of homocysteine with HNC and esophagus cancer risk was present both among non-alcohol consumers and non-smokers (Fig. 1), suggesting that residual confounding by these risk factors does not explain the association with risk. Further, while the association of homocysteine risk was particularly evident for HNC cancers that were diagnosed within three years after blood draw (Fig. 1), it was clearly present during the whole follow-up period (Fig. 1, Supporting Information Table 4). This observation indicates that diagnosis related behavioral or pre-malignant changes that may affect circulating biomarkers close to diagnosis does not explain the overall association of homocysteine with risk, i.e. as would be expected by reverse causality. We also compared homocysteine levels of HNC cases with an alternative control

Table 3. Odds ratios and 95% confidence intervals of upper aero-digestive tract (UADT) cancer for plasma levels of vitamins B2, B6, folate, B12, homocysteine and methionine by tumour sites[1]

		Odds ratio (95% confidence interval)							
Quartile (range)	Control (n = 995)	Esophagus adenocarcinoma cases (n = 74)		Larynx and hypopharynx cases (n = 144)[2]		Oral cavity and Gum cases (n = 108)[2]		Oropharynx cases (n = 67)[2]	
Riboflavin (vitamin B2), nmol/L[3]									
1 (2.5–9.4)	244 (24.6%)	11 (14.9%)	1 [Reference]	55 (37.9%)	1 [Reference]	29 (26.9%)	1 [Reference]	21 (31.3%)	1 [Reference]
2 (9.5–13.1)	237 (23.8%)	15 (20.3%)	1.36 (0.58–3.20)	29 (20%)	0.61 (0.35–1.08)	23 (21.3%)	1.01 (0.54–1.90)	11 (16.4%)	0.52 (0.22–1.22)
3 (13.2–21.3)	272 (27.3%)	22 (29.7%)	1.52 (0.67–3.45)	42 (29%)	0.92 (0.54–1.56)	31 (28.7%)	1.47 (0.80–2.72)	19 (28.4%)	1.04 (0.50–2.17)
4 (21.4–199)	242 (24.3%)	26 (35.1%)	1.95 (0.84–4.52)	19 (13.1%)	0.75 (0.39–1.44)	25 (23.1%)	1.63 (0.83–3.22)	16 (23.9%)	1.24 (0.55–2.78)
p for trend[4]			0.21		0.85		0.16		0.38
Pyridoxal 5′-phosphate (Vitamin B6), nmol/L[3]									
1 (7.2–25.6)	259 (26%)	23 (31.1%)	1 [Reference]	44 (30.3%)	1 [Reference]	41 (38%)	1 [Reference]	22 (32.8%)	1 [Reference]
2 (25.7–34.5)	250 (25.2%)	18 (24.3%)	0.81 (0.40–1.61)	34 (23.4%)	1.00 (0.56–1.78)	29 (26.9%)	0.86 (0.49–1.50)	11 (16.4%)	1.12 (0.51–2.47)
3 (34.6–47.6)	232 (23.3%)	17 (23%)	0.88 (0.42–1.82)	28 (19.3%)	1.16 (0.62–2.14)	23 (21.3%)	0.80 (0.44–1.47)	19 (28.4%)	1.34 (0.59–3.06)
4 (47.7–272)	254 (25.5%)	16 (21.6%)	0.63 (0.30–1.33)	39 (26.9%)	1.34 (0.75–2.41)	15 (13.9%)	0.47 (0.24–0.91)	15 (22.4%)	2.30 (1.08–4.94)
p for trend[4]			0.58		0.65		0.17		0.05
Serum folate (vitamin B9), nmol/L[3]									
1 (0.3–9.1)	269 (27.1%)	22 (29.7%)	1 [Reference]	43 (29.7%)	1 [Reference]	43 (39.8%)	1 [Reference]	20 (29.9%)	1 [Reference]
2 (9.2–12.8)	254 (25.6%)	13 (17.6%)	0.8 (0.36–1.80)	32 (22.1%)	1.02 (0.57–1.82)	22 (20.4%)	0.65 (0.36–1.18)	14 (20.9%)	0.79 (0.37–1.71)
3 (12.9–18.1)	223 (22.4%)	13 (17.6%)	0.86 (0.37–2.00)	43 (29.7%)	2.01 (1.12–3.60)	23 (21.3%)	0.84 (0.46–1.54)	14 (20.9%)	1.02 (0.47–2.21)
4 (18.2–109)	248 (24.9%)	26 (35.1%)	1.68 (0.79–3.56)	27 (18.6%)	1.05 (0.56–1.98)	20 (18.5%)	0.62 (0.33–1.18)	19 (28.3%)	1.23 (0.59–2.58)
p for trend[4]			0.24		0.99		0.04		0.77
Cobalamin (Vitamin B12), pmol/L[3]									
1 (75.1–265)	239 (24%)	20 (27%)	1 [Reference]	43 (29.7%)	1 [Reference]	37 (34.3%)	1 [Reference]	20 (29.9%)	1 [Reference]
2 (266–328)	247 (24.8%)	23 (31.1%)	1.27 (0.64–2.52)	34 (23.4%)	0.89 (0.50–1.56)	27 (25%)	0.67 (0.38–1.20)	16 (23.9%)	0.94 (0.46–1.95)
3 (329–391)	228 (22.9%)	13 (17.6%)	0.79 (0.36–1.73)	28 (19.3%)	0.69 (0.38–1.25)	18 (16.7%)	0.50 (0.26–0.95)	14 (20.9%)	0.90 (0.42–1.93)
4 (392–2737)	280 (28.2%)	18 (24.3%)	1.17 (0.56–2.44)	40 (27.6%)	1.00 (0.57–1.74)	26 (24.1%)	0.64 (0.35–1.15)	17 (25.3%)	0.93 (0.44–1.96)
p for trend[4]			0.40		0.78		0.53		0.80
Homocysteine, μmol/L[3]									
1 (4.9–8.3)	269 (27.1%)	10 (13.5%)	1 [Reference]	32 (22.1%)	1 [Reference]	19 (17.6%)	1 [Reference]	11 (16.4%)	1 [Reference]
2 (8.4–10.1)	238 (23.9%)	16 (21.6%)	1.35 (0.57–3.22)	34 (23.4%)	0.97 (0.53–1.79)	28 (25.9%)	1.64 (0.86–3.14)	16 (23.9%)	1.66 (0.72–3.84)
3 (10.2–12.4)	251 (25.3%)	19 (25.7%)	0.92 (0.38–2.18)	32 (22.1%)	0.67 (0.36–1.23)	18 (16.7%)	0.96 (0.46–2.00)	15 (22.4%)	1.91 (0.79–4.62)
4 (12.5–64.9)	236 (23.7%)	29 (39.2%)	1.19 (0.51–2.77)	47 (32.4%)	1.23 (0.67–2.26)	43 (39.8%)	2.45 (1.26–4.79)	25 (37.3%)	3.26 (1.40–7.60)
p for trend[4]			0.72		0.33		8×10^{-4}		0.008

Table 3. Odds ratios and 95% confidence intervals of upper aero-digestive tract (UADT) cancer for plasma levels of vitamins B2, B6, folate, B12, homocysteine and methionine by tumour sites[1] (Continued)

		Odds ratio (95% confidence interval)					
Quartile (range)	Control (n = 995)	Esophagus adenocarcinoma cases (n = 74)		Larynx and hypopharynx cases (n = 144)[2]		Oral cavity and Gum cases (n = 108)[2]		Oropharynx cases (n = 67)[2]	
Methionine, μmol/L[3]									
1 (12.6–21.8)	265 (26.6%)	21 (28.4%)	1 [Reference]	38 (26.2%)	1 [Reference]	31 (28.7%)	1 [Reference]	28 (41.8%)	1 [Reference]
2 (21.9–25.1)	255 (25.6%)	13 (17.6%)	0.87 (0.4–1.89)	32 (22.1%)	0.82 (0.45–1.47)	24 (22.2%)	0.77 (0.42–1.42)	16 (23.9%)	0.67 (0.34–1.32)
3 (25.2 - 29.3)	252 (25.4%)	23 (31.1%)	1.73 (0.87–3.47)	37 (25.5%)	0.92 (0.51–1.65)	31 (28.7%)	1.05 (0.58–1.89)	11 (16.4%)	0.50 (0.23–1.10)
4 (29.4–62.9)	223 (22.4%)	17 (23%)	1.32 (0.63–2.80)	38 (26.2%)	1.08 (0.6–1.96)	22 (20.4%)	0.81 (0.42–1.54)	12 (17.9%)	0.56 (0.26–1.22)
p for trend[4]		0.21		0.53		0.57		0.01	

Assessed by analysing cancer cases of the head and neck and esophagus, and all controls combined by unconditional logistic regression, adjusting for country, sex, age at recruitment (in 5-year groups), educational attainment (in four groups), smoking status (never/former/current/missing), cotinine (quartiles defined among current smokers) and alcohol intake at recruitment (g/day). [2]Adenocarcinoma excluded. [3]Quartile cut-off points were determined based on the plasma level distribution of each biomarker among the 516 individually matched controls of the head and neck and esophagus. [4]p for trend assessed by the base 2 logarithm of the serum levels.

series in an unconditional analysis and the results on risk were consistent with that of the conditional analysis, thus further highlighting the consistency of the overall findings. In contrast, no convincing associations were observed between pre-diagnostically measured one-carbon metabolism biomarkers and all-cause mortality among cases, nor for cause-specific mortality considering events where HNC or ESCC was indicated as underlying cause of death (data not shown). Evaluating other specific causes of death, e.g. cardiovascular disease, was not feasible due to few relevant. Furthermore, it would have been informative to attain blood samples at diagnosis to fully evaluate the importance of these biomarkers for HNC survival.

Potential roles of homocysteine in carcinogenesis

Homocysteine is a thiol-containing amino acid produced through the catabolism of methionine.[27] Experiencing very high blood levels of homocysteine is a condition typically referred to as hyperhomocysteinemia, a well-established risk indicator for cardiovascular disease.[28–30] While randomized trials have shown that folate supplementation is an efficient means to reduce circulating homocysteine levels, it does not seem to translate into reduced risk of subsequent cardiovascular disease.[31–33] The importance of homocysteine in carcinogenesis is not clear. Homocysteine can be re-methylated to methionine by receiving a methyl group from folate, a reaction catalyzed by the vitamin B12-dependant methionine synthase. Some indication of carcinogenic effects of homocysteine have been provided by *in vitro* studies in which high homocysteine levels have been associated with increasing proliferation rates and oxidative stress.[34,35]

Previous studies investigating the association between circulating homocysteine and other cancer sites have not provided consistent results on risk. For instance, some prospective cohort studies have reported that elevated homocysteine was associated with increased risk of colorectal adenoma,[36–39] but not increased risk of subsequent colorectal cancer (CRC).[40–43] However, the largest and most recent prospective study on CRC indicated that high circulating homocysteine was associated with around 50% increased risk.[44] In our previous investigation of lung cancer,[12] we initially noted a positive association between homocysteine and risk in an unadjusted analysis, consistent with the well-established reverse relationship between folate and homocysteine. However, in contrast to the current study, tobacco smoking fully accounted for the association of homocysteine with risk in subsequent adjusted analysis (only the smoking adjusted analysis was reported).

Importantly, because folate deficiency leads to accumulation of homocysteine we cannot exclude the possibility that the positive association of homocysteine with risk observed in the current study indicates an underlying inverse relation between folate and risk (Supporting Information Table 2). In conditional analysis we observed a nominal inverse association between folate and risk that was primarily driven by an

increase in risk among subjects in the bottom quartile compared to those in quartiles 2 to 4. This observation would seem consistent with the inverse relation between folate and homocysteine, and that the increase in risk of homocysteine was primarily seen among subjects in the top quartile. Indeed, mutually adjusted conditional analysis (data not shown) indicated that these risk associations were not independent, homocysteine accounting for the association of folate, but not vice-versa. Nonetheless, the association between folate and HNC risk seen in conditional analysis was not supported by a comparison of the cases with the alternative control series. This result would not seem to provide strong support for a decrease in risk in subjects with high dietary intake of folate overall as seen in some previous studies,[45,46] nor in subgroups defined by head and neck cancer risk factors (Supporting Information Fig. 4).

Evaluating if the notably stronger and consistent risk association of homocysteine than of folate is reproducible will require an additional, well-powered prospective analysis on HNC and ESCC, a study that may not be feasible a single cohort given the rarity of the disease. Studies of relevant gene variants that are proxies for true exposures[47] may also provide information on the importance of maintaining adequate folate and moderate homocysteine levels over the life course along the lines of Mendelian randomization.[48]

Conclusions

This study indicates that subjects with high levels of homocysteine are at increased risk of developing squamous cell carcinoma of the head and neck. Further large-scale prospective studies are warranted to confirm the robustness of homocysteine as an indicator of future risk, and its potential causal role in the pathogenesis of these cancers.

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Author contributions

M.J. and P.B. initiated, acquired the main funding, and designed this investigation. P.M.U., S.E.V. and Ø.M. led the laboratory analysis. A.F. conducted the statistical analysis under supervision of M.J. A.F. and M.J. drafted the first version of the manuscript with important contributions from P.B. All authors were involved with collection of data, data interpretation, critical revisions of the article, and approval of the final version. M.J. 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. E.R. is the overall coordinator of the EPIC study which he designed and implemented in collaboration with the main investigators in the collaborating centers.

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