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Dietary folate intake and *K-ras* mutations in sporadic colon and rectal cancer in The Netherlands Cohort Study

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We studied the association between dietary folate and specific *K-ras* mutations in colon and rectal cancer in The Netherlands Cohort Study on diet and cancer. After 7.3 years of follow-up, 448 colon and 160 rectal cancer patients and 3,048 sub-cohort members (55–69 years at baseline) were available for data analyses. Mutation analysis of the *K-ras* gene was carried out on all archival adenocarcinoma specimens. Case–cohort analyses were used to compute adjusted incidence rate ratios (RR) and 95% confidence intervals (CI) for colon and rectal cancer overall and for *K-ras* mutation status subgroups according to 100 μg/day increased intake in dietary folate. Dietary folate intake was not significantly associated with colon cancer risk for men or women, neither overall nor with *K-ras* mutation status. For rectal cancer, folate intake was associated with a decreased disease risk in men and was most pronounced for *K-ras* mutated tumors, whereas an increased association was observed for women. Regarding the *K-ras* mutation status in women, an increased association was observed for both wild-type and mutated *K-ras* tumors. Specifically, folate intake was associated with an increased risk of G>T and G>C transversions in rectal tumors (RR = 2.69, 95% CI = 1.43–5.09), but inversely associated with G>A transitions (RR = 0.08, 95% CI = 0.01–0.53). Our data suggest that the effect of folate on rectal cancer risk is different for men and women and depends on the *K-ras* mutation status of the tumor.
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Key words: men; women; transversion; wild-type

To date, results from epidemiological studies[1–4] generally show an inverse association between dietary folate and the risk of colorectal cancer. Associations may be different, however, for men *vs.* women and for colon *vs.* rectum.[4,5] In addition, the results from most animal studies support the epidemiological data, but results were not always consistent.[1,2]

Folate plays an important role in the one-carbon metabolism, a series of interrelated biochemical reactions involved in DNA synthesis and methylation of DNA, RNA and proteins[6] in animals and humans. Two pathways have been described linking a low folate status to colon and rectum carcinogenesis.[6,7] First, a deficient intracellular form of folate, 5,10-methylenetetrahydrofolate (5,10-methyleneTHF), leads to a decreased availability of methyl groups for uracil that may result in less available thymine. Eventually, the increased uracil incorporation in DNA may lead to DNA strand breaks and chromosome instability. Second, sub-optimal folate status may result in global DNA hypomethylation and regional hypermethylation in gene promoters that interferes with gene expression and can impair DNA repair.[8,9] DNA strand breaks, global DNA hypomethylation and regional promoter hypermethylation are common events in colon cancer.

The association between folate and colorectal cancer was evaluated earlier in The Netherlands Cohort Study on diet and cancer (NLCS), based on 7.3 years of follow-up.[3,10] In this study,[3] the association between high folate intake and colon cancer tended to be inverse for men and women after the exclusion of the first year of follow-up, but did not reach statistical significance. For rectal cancer, folate was inversely associated with the disease risk in men, although borderline significant, but not in women. Evaluating these findings, it would be interesting to study the association between dietary folate and CRC when specific mutations in genes involved in colorectal carcinogenesis are taken into account.

The oncogenic activation of *K-ras* by mutations is a genetic alteration that occurs in adenomas (10%) as well as in carcinomas (40%) in colon and rectal cancer. Activating mutations are mainly found in codons 12 and 13.[11–14] The types of point mutations observed most frequently are G>A transitions, and G>T and G>C transversions.[14–16] Possibly, the G>A transitions observed frequently in the *K-ras* gene are a result of impaired promoter methylation of the DNA-repair gene O^6-methylguanine DNA methyltransferase (MGMT)[9] that could, in part, be due to a low folate status.[10]

Only 3 epidemiological studies report on the association between folate intake and *K-ras* mutation status in colon (rectal) tumors[17,18] and in colorectal adenomas[7] with varying results. It is difficult to compare the results from different studies, however, due to differences in case groups and reference groups. In our current study, the association between dietary folate intake and the risk of specific point mutations in the *K-ras* oncogene in patients with colon and rectal cancer were studied for men and women within the framework of the NLCS.

Material and methods

Study population

The participants in our study are incident colon and rectal cancer cases and subcohort members from the NLCS, which has been described in detail elsewhere.[19] The study was initiated in 1986 and includes 58,279 men and 62,573 women, 55–69 years of age at baseline, who originated from 204 Dutch municipalities with computerized population registries. A self-administered questionnaire on diet and other risk factors for cancer was completed at baseline. The entire cohort is being monitored for cancer occurrence by annual record linkage to the Netherlands Cancer Registry (NCR; 9 cancer registries in The Netherlands) and to PALGA, a nationwide network and registry of histo- and cytopathology reports (www.palga.nl).[20] In the municipalities included in the NLCS, the NCR and PALGA have nearly 100% coverage since the start of the study.[21–24] PALGA also provides necessary information on the identification of the pathology laboratory location of the storage of paraffin-embedded blocks of the eligible CRC patients. Accumulation of person-time in the cohort has been estimated through biennial vital status follow-up of a subcohort of 3,500 men and women who were randomly selected after baseline exposure measurement. Cases with prevalent cancer other than

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non-melanoma skin cancer were excluded from the subcohort, which left 3,346 men and women for analysis.

The first 2.3 years of follow-up were excluded because of possible preclinical disease affecting exposure status and because of incomplete coverage of PALGA alone in some of the municipalities included in the NLCS. Within this period, 83 subcohort members were either deceased or diagnosed with cancer other than non-melanoma skin cancer, leaving 3,263 men and women for analysis. From 1989–1994, 929 incident cases with histologically confirmed CRC were observed of whom 819 could also be linked to a PALGA report of the lesion. The PALGA database was used to identify and locate tumor tissue in Dutch pathology laboratories. CRC was classified according to site as follows: colon *i.e.*, cecum through sigmoid colon (ICD-O-1 codes: 153.0, 153.1, 153.2, 153.3, 153.4, 153.5, 153.6, 153.7, 153.8, 153.9), rectosigmoid (ICD-O-1 code 154.0) and rectum (ICD-O-1 code 154.1). Information about age at baseline, gender and family history of CRC (at baseline) was retrieved from the NLCS database.

Tissue samples

Tumor material of all CRC patients was collected after approval by the Medical Ethics Committees (MEC) of Maastricht University, the NCR and PALGA.[14] Subsequently, all pathology laboratories in The Netherlands agreed to make relevant tissue samples available upon request from PALGA. Tissue samples of the 819 cases present in 54 pathology laboratories throughout The Netherlands, were collected between August 1999 and December 2001. For 43 (5%) untraceable tumor tissue specimens and 39 (5%) specimens with insufficient CRC tumor material, *K-ras* mutation status could not be determined. Tumor material from 737 incident colorectal adenocarcinoma cases was available of whom 476 were colon cancer cases, 85 were rectosigmoid cancer cases and 176 were rectal cancer cases. Statistical analyses were carried out separately for colon and rectal cancer as differences in the etiology of colon and rectal cancer have been reported.[25] Because the rectosigmoid can be considered as a clinically applied term rather than an anatomically defined transitional zone between the colon and rectum, patients with a rectosigmoid tumor were excluded from data-analyses. Moreover, the number of patients with a rectosigmoid tumor was too small for adequate stratified analyses.[14]

Detection of K-ras *mutations*

Mutation analysis of the exon 1 fragment of the *K-ras* oncogene, spanning codons 8–29, was carried out on archival colorectal adenocarcinoma specimens of all 737 CRC patients using macrodissection, nested PCR and direct sequencing of purified fragments, which has been described in detail elsewhere.[14]

Food frequency questionnaire

The dietary section of the questionnaire was a 150-item semiquantitative food frequency questionnaire, which concentrated on habitual consumption of food and beverages during the year preceding the start of the study. Daily mean nutrient intakes were calculated by cumulating the multiplied frequencies and portion sizes of all food items with their tabulated nutrient contents from the Dutch food composition table.[26] The questionnaire was validated against a 9-day diet record.[27] Questionnaire data were key-entered twice and processed for all incident cases in the cohort and for all subcohort members in a manner blinded with respect to case/subcohort status. This was done to minimize observer bias in coding and interpretation of the data.

Folate data were derived from a validated liquid chromatography trienzyme method[28] used to analyze the 125 most important dutch foods contributing to folate intake.[29] The mean daily intakes of all other relevant nutrients were calculated using the computerized Dutch Food Composition Table (NEVO Table, 1986).[26] For data-analyses, an increment of 100 μg/day increase in folate intake for men and women was computed.

For 257 subjects (28 incident colon adenocarcinoma cases, 16 incident rectal adenocarcinoma cases and 215 subcohort members, two subcohort members were also colon or rectal cancer cases), dietary data were incomplete or inconsistent, and they were excluded from the analyses. These subjects either (*i*) left 60 or more (of 150) questionnaire items blank and ate fewer than 35 items at least once per month or (*ii*) left one or more item blocks (groups of items, *e.g.*, beverages) blank. Additional details are given elsewhere.[27] Hence, 608 colon (242 men and 206 women) and rectal cases (104 men and 56 women) and 3,048 subcohort members (1,475 men and 1,573 women) were available for data-analyses.

Daily intake of dietary fibre (g/day), vitamin B_2 (mg/day), vitamin B_6 (mg/day), vitamin C (μg/day), iron (mg/day), alcohol (g/day), fresh meat (g/day), and total energy (kcal/day) and age at baseline (years), Body Mass Index (BMI; kg/m_2), physical activity ($<$30 min/day, 30–60 min/day, 60–90 min/day, $>$90 min/day), family history of CRC (yes/no) and smoking status (never/ex/current) were regarded as potential confounders.

Statistical analysis

Analyses were conducted for men and women separately. The overall frequency of *K-ras* mutations as well as the type of mutation were computed for all colon and rectal cancer cases as described elsewhere.[14] Mean values of the continuous variables age at baseline (years), intake of total folate, dietary fiber, alcohol, fresh meat, vegetables and total energy and BMI were evaluated for subcohort members and colon and rectal cancer cases with wild-type and mutated *K-ras* gene. Differences in mean values of the continuous variables between patients with wild-type and mutated *K-ras* gene were tested with the Student's *t*-test or the Mann-Whitney *U*-test if the variables were not distributed normally. Distributions of the categorical variables family history of CRC, smoking status and physical activity were evaluated for subcohort members and colon and rectal cancer patients with wild-type and mutated *K-ras* gene and tested for differences between patient groups with the χ^2 test. The statistical software package SPSS (version 9) was used for these analyses.

Age-adjusted and multivariate-adjusted incidence rate ratios (RR) and their 95% confidence intervals (CI) for colon and rectal cancer risk were calculated for men and women separately, using Cox proportional hazards regression models with the STATA statistical software package (intercooled STATA, version 7). Standard errors were estimated using the robust Huber-White sandwich estimator to account for additional variance introduced by sampling from the cohort. This method is equivalent to the variance-covariance estimator as presented by Barlow.[30] The proportional hazards assumption was tested using the scaled Schoenfeld residuals.[31] Those variables that were found to contribute substantially ($p < 0.10$) to the multivariate model for colon or rectal cancer (age, smoking, BMI, total fresh meat consumption, and intake of iron and vitamin C) were included as covariates in all multivariate analyses. Additional adjustment for dietary fiber and energy intake was carried out, in line with the previous report on folate and overall colon and rectal cancer risk in the Netherlands Cohort Study.[3] The person-years at risk, estimated from the subcohort, were used in the analyses.

To date, foods are not fortified with folic acid in The Netherlands. For the time being, an intake of 200 g of vegetables and 2 pieces of fruit daily is recommended. This is approximately twice the amount of fruit and vegetables found to be consumed in the Dutch National Food Consumption Survey (DNFCS) carried out in 1992. The fruit and vegetable intake accounts for approximately 50 μg folate.[29] We calculated RR according to an increase in intake of 100 μg/day of folate. Approximately twice this additional amount would be necessary to reach the United States recommended daily allowance of 400 μg/day in the majority of the men and women of this cohort.

Results

The overall frequency and spectrum of mutations in the *K-ras* gene have been presented in detail elsewhere.[14] In brief, 227 mutations was found in 218 of 608 (36%) colon and rectal cancer patients. The mutations observed most frequently are the G>A transitions (54%), G>T transversions (33%) and G>C transversions (7%). The observed frequencies of the mutations in this series of patients are similar to the frequencies of the 737 colorectal cancer cases (including the rectosigmoid) for whom *K-ras* status was determined.[14]

Table I shows the folate intake and other baseline characteristics for colon cancer cases and subcohort members, separately for men and women. The mean age of the subcohort members was 61.3 years for men and 61.4 years for women. Overall, colon cancer cases were older than the subcohort members. Colon cancer patients with a mutated *K-ras* gene were significantly older than the patients with a wild-type *K-ras* gene (p-values 0.04 and 0.06 for men and women, respectively). Dietary folate intake was higher among subcohort members as compared to the different colon cancer subgroups. No statistically significant differences for other (dietary) factors were observed for colon cancer cases without and with a *K-ras* mutation in men and women as presented in Table I.

For rectal cancer cases and subcohort members, the mean folate intake and other baseline characteristics are presented in Table II. Rectal cancer patients were older than the subcohort members. Dietary folate intake among subcohort members and the different rectal cancer subgroups was similar. Women with a mutated *K-ras* rectal tumor were more often ex-smoker as compared to women with a wild-type *K-ras* rectal tumor and subcohort members. No statistically significant differences for other (dietary) factors were observed for rectal cancer cases without and with a *K-ras* mutation in men and women as presented in Table II.

For colon cancer, associations between folate intake and risk of disease, as well as *K-ras* mutation status are presented for men and women in Table III. No significant association was observed between dietary folate and overall colon cancer risk or with the *K-ras* mutation status after adjustment for age, BMI, smoking, alcohol, fresh meat, energy intake and family history of colorectal cancer, iron intake, vitamin C intake and dietary fibre.

For rectal cancer, associations between folate intake and risk of disease overall, as well as *K-ras* mutation status are presented for men and women in Table IV. A significant, inverse association was observed between folate and overall rectal cancer risk in men (RR = 0.58, 95% CI = 0.36–0.93), as well as with a *K-ras* mutation in the tumor (RR = 0.40, 95% CI = 0.17–0.89). The association was strongest for G>T or G>C transversions in the *K-ras* gene (RR = 0.19, 95% CI = 0.03–1.07). For women, a significant positive association was observed between folate and overall rectal cancer risk (RR = 1.85, 95% CI = 1.13–3.02) and this association was observed for rectal tumors without and with a *K-ras* oncogene mutation (RR = 1.94, 95% CI = 1.03–3.66 and 1.83, 95% CI = 0.91–3.68, respectively). After taking into account the specific point mutations in the *K-ras* gene, an increased risk was observed for rectal tumors harboring G>T or G>C transversions (RR = 2.69, 95% CI = 1.43–5.09). A significant inverse association was observed between folate and G>A transitions (RR = 0.08, 95% CI = 0.01–0.53).

Discussion

In a large cohort with 448 incident colon cancer patients and 160 incident rectal cancer patients, no significant associations were observed between folate intake and the risk of colon cancer in men and women, neither overall nor after the *K-ras* mutation status was taken into account. For men, a protective effect was observed for folate and rectal cancer risk, overall and most pronounced, with *K-ras* mutated tumors. For women, however, a positive association was observed between folate and rectal cancer risk, overall and also when the *K-ras* mutation status was taken into account. Regarding the specific point mutations in the *K-ras* gene of rectal tumors in women, dietary folate was positively associated with G>T or G>C transversions, however, inversely associated with G>A transitions.

The current cohort study on folate intake and specific *K-ras* mutations is, to our knowledge, the only prospective study carried out to date taking into account the *K-ras* mutation status. The prospective design of our study and high completeness of follow-up of cancer incidence and subcohort make information and selection bias unlikely. In addition, as a result of the exclusion of the first 2.3 years of follow-up, the chance of information bias due to potential preclinical colorectal cancer is minimal.

Although several epidemiological studies have been conducted on dietary factors and *K-ras* mutations in colon and rectal cancer, only 3 have reported on folate intake, *i.e.*, one large case-control study with colon cancer patients,[17] one case-control study with colon and rectal cancer patients[18] and one cross-sectional case–case study with colorectal adenoma patients.[7] Slattery *et al.*[17] observed a statistically significant, inverse association between dietary folate intake and the risk of colon tumors with a wild-type *K-ras* gene (OR for the lowest *vs.* the highest tertile of intake 1.4, 95% CI = 1.1–1.8; $p_{trend} < 0.01$). In another smaller case-control study with colon and rectal cancer patients, no statistical differences in dietary folate intake as observed between cases with a *K-ras* mutation compared to cases with a wild-type *K-ras*.[18] Therefore, case–control analyses regarding dietary folate intake was not further explored in the study by Bautista *et al.*[18] In a large case–case comparison of colorectal adenoma patients,[7] individuals in the upper tertile of total folate intake (dietary and supplemental folate), compared to individuals in the lowest tertile, had a nearly 50% lower risk of having *K-ras* mutation-positive adenomas (OR = 0.52, 95% CI = 0.30–0.88; $p_{trend} = 0.02$). This reduction in risk of *K-ras* mutation-positive adenomas was not observed for dietary folate intake alone (OR = 0.91, 95% CI = 0.50–1.64; $p_{trend} = 0.75$). Caution is warranted in interpreting these results, because case–case comparisons, including both male and female colon and rectal adenomas, were carried out. Case–case comparisons are valuable for the estimation of etiologic heterogeneity with regard to *K-ras* mutation status. They do not, however, give information on the direction of the association between folate and *K-ras* wild-type or mutated tumors, respectively. This can only be obtained with case–control comparisons. In addition, we observed distinct differences in associations between men and women and colon and rectal cancer. Our results do not support the protective effect of folate for mutated *K-ras* colon tumors observed in the other studies. One explanation could be that dietary folate intake in The Netherlands is too low to evoke its potentially protective effect in colon and rectal cancer, although a protective effect was observed for male rectal cancer. In the NLCS, 22% of the study population used vitamin B supplements in 1986.[32] Due to legislative restrictions in The Netherlands, however, it was not until 1994 that using folic acid in vitamin supplements was allowed. The overall effect of folic acid supplements in this cohort is presumably negligible.

In our current study, an inverse association was observed for women between high folate intake and rectal tumors harboring a G>A transition in the *K-ras* oncogene. This was also observed for both men and women by Slattery *et al.*[17] although not statistically significant, for G>A transitions at the second position of codon 12 (OR for lowest tertile *vs.* highest tertile of intake 0.7, 95% CI = 0.4–1.3; $p_{trend} = 0.11$). This could be an indirect consequence of promotor hypermethylation of mismatch repair genes like the O^6-methylguanine DNA methyltransferase (MGMT) gene. Van Engeland *et al.*[10] evaluated the association between promoter hypermethylation of genes involved in colorectal carcinogenesis, like MGMT, and folate and alcohol intake within the framework of the NLCS. MGMT is a DNA repair protein that removes adducts from the O^6 position of guanine[9] in DNA, thereby preventing G>A or C>T mutations. Despite the limited size of the study carried out by van Engeland *et al.*,[10] the data suggest that low

TABLE I – NUTRIENT INTAKE (MEAN ± SD) AND OTHER CHARACTERISTICS AMONG COLON CANCER CASES AND SUBCOHORT MEMBERS

Characteristic	Men				Women			
	Subcohort	Colon cancer			Subcohort	Colon cancer		
		Wild type *K-ras*	*K-ras* mutation	*p*-value[1]		Wild type *K-ras*	*K-ras* mutation	*p*-value[1]
N	1,475	153	89[2]		1,573	144	62[3]	
Age (years)	61.3 ± 4.2	62.5 ± 4.2	63.7 ± 4.2	0.04	61.4 ± 4.3	62.8 ± 4.1	63.9 ± 3.6	0.06
Folate (μg/day)	224.2 ± 72.0	225.4 ± 62.9	225.1 ± 83.6	0.97	199.8 ± 64.7	189.4 ± 67.3	192.9 ± 61.6	0.72
Other dietary factors								
Alcohol (g/day)	14.5 ± 16.4	15.7 ± 16.4	15.6 ± 15.9	0.95	5.7 ± 9.4	6.0 ± 12.4	3.9 ± 7.0	0.22
Fiber (g/day)	28.7 ± 8.8	28.8 ± 7.2	29.7 ± 8.9	0.40	25.5 ± 7.2	24.4 ± 7.4	24.9 ± 7.9	0.68
Energy (kcal/day)	2,165.6 ± 512.1	2,157.1 ± 452.6	2,069.4 ± 438.6	0.14	1,691.4 ± 408.5	1,660.9 ± 400.7	1,663.5 ± 416.7	0.97
Fresh meat (g/day)	105.9 ± 43.3	101.5 ± 35.9	103.6 ± 36.9	0.66	93.8 ± 40.4	95.2 ± 38.0	93.1 ± 41.6	0.73
Vitamin B6 (mg/day)	1.5 ± 0.4	1.6 ± 0.3	1.6 ± 0.4	0.90	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	0.58
Vitamin C (mg/day)	98.1 ± 41.5	100.9 ± 41.5	108.5 ± 42.9	0.18	108.2 ± 43.8	102.0 ± 47.1	105.7 ± 47.4	0.61
Iron (mg/day)	13.2 ± 3.2	13.5 ± 2.9	13.7 ± 3.3	0.68	11.7 ± 2.7	11.4 ± 2.4	11.2 ± 2.9	0.69
Methionine (g/day)	1.7 ± 0.4	1.7 ± 0.4	1.7 ± 0.4	0.16	1.5 ± 0.4	1.5 ± 0.3	1.5 ± 0.4	0.46
Other characteristics								
BMI (kg/m^2)	25.0 ± 2.6	25.3 ± 2.6	25.9 ± 3.1	0.11	25.1 ± 3.5	25.7 ± 3.7	25.5 ± 3.6	0.78
Family history (% Yes)	5.7	13.7	11.2	0.58	5.5	13.2	6.5	0.16
Smoker (%)								
Never	13.4	11.8	11.2		59.1	63.2	74.2	
Ex smoker	51.3	62.1	67.4		20.2	22.9	16.1	
Current smoker	35.3	26.1	21.3	0.67	20.7	13.9	9.7	0.31
Physical activity (%)[4]								
<30 minutes/day	15.0	10.6	11.4		26.3	29.4	33.3	
30–60 minutes/day	29.7	31.8	27.3		35.4	35.7	40.0	
60–90 minutes/day	34.8	32.5	38.6		27.3	26.6	15.0	
>90 minutes/day	20.4	25.2	22.7	0.76	11.0	8.4	11.7	0.34

[1]Comparing cases with at least one *K-ras* mutation to cases without a *K-ras* mutation.–[2]2 out of 89 male colon cancer cases had more than one *K-ras* gene mutation.–[3]4 out of 62 female colon cancer cases had more than one *K-ras* gene mutation.–[4]For 41 subcohort members and 6 colon cancer cases, information on physical activity was not available.

TABLE II – NUTRIENT INTAKE (MEAN ± SD) AND OTHER CHARACTERISTICS AMONG RECTAL CANCER CASES AND SUBCOHORT MEMBERS

	Men				Women			
	Subcohort	Rectal cancer			Subcohort	Rectal cancer		
		Wild-type *K-ras*	*K-ras* mutation	*p*-value[1]		Wild-type *K-ras*	*K-ras* mutation	*p*-value[1]
N	1,475	67	37[2]		1,573	26	30[3]	
Age (years)	61.3 ± 4.2	62.3 ± 4.2	62.4 ± 4.1	0.89	61.4 ± 4.3	63.5 ± 4.0	62.0 ± 3.9	0.17
Folate (μg/day)	224.2 ± 72.0	222.4 ± 52.5	212.3 ± 67.6	0.40	199.8 ± 64.7	212.2 ± 70.7	216.2 ± 94.8	0.86
Other dietary factors								
Alcohol (g/day)	14.5 ± 16.4	16.9 ± 19.3	14.7 ± 12.5	0.53	5.7 ± 9.4	5.3 ± 6.5	6.6 ± 10.3	0.60
Fiber (g/day)	28.7 ± 8.8	29.2 ± 8.3	29.9 ± 9.1	0.67	25.5 ± 7.2	24.2 ± 5.9	24.6 ± 4.5	0.74
Energy (kcal/day)	2,165.6 ± 512.1	2,182.6 ± 483.2	2,165.0 ± 467.0	0.86	1,691.4 ± 408.5	1,627.0 ± 370.6	1,789.7 ± 328.4	0.09
Fresh meat (g/day)	105.9 ± 43.3	101.3 ± 38.6	92.8 ± 40.2	0.30	93.8 ± 40.4	98.8 ± 45.2	99.2 ± 32.0	0.97
Vitamine B6 (mg/day)	1.5 ± 0.4	1.5 ± 0.4	1.5 ± 0.4	0.91	1.3 ± 0.3	1.4 ± 0.3	1.4 ± 0.3	0.88
Vitamine C (mg/day)	98.1 ± 41.5	108.5 ± 46.8	105.0 ± 56.9	0.74	108.2 ± 43.8	109.5 ± 43.1	111.3 ± 45.8	0.88
Iron (mg/day)	13.2 ± 3.2	13.6 ± 2.9	13.3 ± 3.1	0.83	11.7 ± 2.7	11.6 ± 2.2	11.6 ± 2.2	0.95
Methionine (g/day)	1.7 ± 0.4	1.7 ± 0.4	1.6 ± 0.4	0.20	1.5 ± 0.4	1.6 ± 0.4	1.5 ± 0.3	0.63
Other characteristics								
BMI (kg/m^2)	25.0 ± 2.6	24.9 ± 2.5	25.1 ± 2.7	0.66	25.1 ± 3.5	25.1 ± 3.5	26.0 ± 3.1	0.27
Family history (% Yes)	5.7	7.5	16.2	0.17	5.5	15.4	6.7	0.29
Smoker (%)								
Never	13.4	9.0	13.5		59.1	69.2	60.0	
Ex smoker	51.3	55.2	54.1		20.2	7.7	30.0	
Current smoker	35.3	35.8	32.4	0.76	20.7	23.1	10.0	0.07
Physical activity (%)[4]								
<30 minutes/day	15.0	9.1	16.7		26.3	42.3	30.0	
30–60 minutes/day	29.7	27.3	30.6		35.4	26.9	23.3	
60–90 minutes/day	34.8	37.9	30.6		27.3	19.2	40.0	
>90 minutes/day	20.4	25.8	22.2	0.64	11.0	11.5	6.7	0.39

[1]Comparing cases with at least one *K-ras* mutation to cases without a *K-ras* mutation.–[2]2 out of 37 male rectal cancer cases had more than one *K-ras* gene mutation.–[3]1 out of 30 female rectal cancer cases had more than one *K-ras* gene mutation.–[4]For 41 subcohort members and 2 rectal cancer cases, information on physical activity was not available.

TABLE III – INCIDENCE RR AND 95% CI FOR COLON CANCER PATIENTS OVERALL AND WITH A (SPECIFIC) *K-RAS* MUTATION STATUS (N = 448) ACCORDING TO DIETARY FOLATE INTAKE

	Men		Women	
	n	RR (95% CI)	*n*	RR (95% CI)
Colon cancer				
RR_{age}	231	1.08 (0.91–1.30)	199	0.87 (0.67–1.15)
$RR_{multivariate}$[1]	231	0.87 (0.66–1.14)	199	0.98 (0.62–1.56)
Wild-type *K-ras*				
$RR_{multivariate}$[1]	148	0.94 (0.69–1.27)	141	0.89 (0.52–1.52)
Mutated *K-ras*				
$RR_{multivariate}$[1]	83	0.76 (0.47–1.22)	58	1.22 (0.52–2.82)
G>A transition				
$RR_{multivariate}$[1]	47	0.78 (0.40–1.53)	34	1.29 (0.48–3.49)
G>T, G>C transversions				
$RR_{multivariate}$[1]	29	0.77 (0.41–1.45)	25	1.56 (0.49–4.95)

[1]RR adjusted for age, BMI, smoking, alcohol, fresh meat, energy intake and family history of CRC, vitamin C, iron, fibre. RR per increment of 100 μg/day increase of dietary folate intake.

TABLE IV – INCIDENCE RR AND 95% CI FOR RECTAL CANCER PATIENTS OVERALL AND WITH A (SPECIFIC) *K-RAS* MUTATION STATUS (N = 160) ACCORDING TO DIETARY FOLATE INTAKE

	Men		Women	
	n	RR (95% CI)	*n*	RR (95% CI)
Rectal cancer				
RR_{age}	99	0.89 (0.68–1.16)	51	1.43 (0.98–2.08)
$RR_{multivariate}$[1]	99	0.58 (0.36–0.93)	51	1.85 (1.13–3.02)
Wild-type *K-ras*				
$RR_{multivariate}$[1]	63	0.69 (0.40–1.19)	24	1.94 (1.03–3.66)
Mutated *K-ras*				
$RR_{multivariate}$[1]	36	0.40 (0.17–0.89)	27	1.83 (0.91–3.68)
G>A transition				
$RR_{multivariate}$[1]	22	0.68 (0.36–1.28)	11	0.08 (0.01–0.53)
G>T, G>C transversions				
$RR_{multivariate}$[1]	13	0.19 (0.03–1.07)	17	2.69 (1.43–5.09)

[1]RR adjusted for age, BMI, smoking, alcohol, fresh meat, energy intake and family history of CRC, vitamin C, iron, fibre. RR per increment of 100 μg/day increase of dietary folate intake.

folate and high alcohol intakes may be associated with promoter hypermethylation in genes involved in CRC (*i.e.*, MGMT). No clear associations were observed for folate and G>A transitions in men, neither in the colon nor the rectum. More etiological insight in the underlying mechanisms relating the differences in tumor site, gender and *K-ras* mutation status in colorectal cancer is required to clarify this issue. Moreover, due to the overall small number of cases when studying subgroups of *K-ras* mutated tumors, the results of our study should be interpreted with caution, and need replication in future larger studies.

We observed an increased risk of rectal cancer with increased folate intake in women. This was observed for both wild-type and mutated *K-ras* gene (*i.e.*, G>T and G>C transversions) but not for G>A transitions as described above. For men, however, an inverse association with G>T and G>C transversions in rectal tumors was observed. Another recent study also showed elevated risks for rectal cancer in women with increased dietary folate intake, though not statistically significant.[4] With the folate food fortifications and extensive use of (multi)vitamin supplements including folate in different countries it is important to further investigate this issue in prospective studies.

In conclusion, in our study dietary folate is not associated significantly with colon cancer risk, neither overall nor with the *K-ras* mutation status. Our data suggest that the effect of folate on rectal cancer risk is different for men and women and depends on the *K-ras* mutation status of the tumor.

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