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Diet, lifestyle and risk of K-ras mutation-positive and -negative colorectal adenomas

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K-ras mutation-positive (K-ras⁺) and -negative (K-ras⁻) colorectal adenomas may differ clinically and pathologically. As environmental compounds may cause mutations in the growth-related K-ras oncogene or affect clonal selection depending on mutational status, we evaluated whether the aetiology of K-ras⁺ and K-ras⁻ adenomas differs. K-ras mutations in codons 12 and 13 were assessed in colorectal adenoma tissue (K-ras⁺: *n* = 81, K-ras⁻: *n* = 453). Dietary and lifestyle data were collected through questionnaires that were also administered to 709 polyp-free controls. Multiple logistic regression analyses showed that intake of vitamin B2 and monounsaturated fat were differently associated with risk of K-ras⁺ and K-ras⁻ adenomas; vitamin B2 was inversely associated with K-ras⁻ (highest vs. lowest tertile: odds ratio (OR) = 0.70, 95% confidence interval (CI) = 0.50–0.97, *p* trend = 0.020), but not with K-ras⁺ adenomas, and a positive association with monounsaturated fat was confined to K-ras⁻ adenomas (OR = 1.57, 95% CI = 1.06–2.34, *p* trend = 0.029). Besides, potential, not statistically significant, differences in risk arose because red meat was distinctly positively associated with K-ras⁺ adenomas (OR = 1.70, 95% CI = 0.94–3.09, *p* trend = 0.061); total dietary and polyunsaturated fat tended to be inversely associated with risk of K-ras⁺ but not of K-ras⁻ adenomas; inverse associations with dairy products, calcium, protein and tea were confined to K-ras⁻ adenomas, and smoking was more markedly positively associated with K-ras⁺ adenomas. No differences in risk of K-ras⁺ and K-ras⁻ adenomas could be detected for other factors. In conclusion, dietary and lifestyle factors may influence risk of K-ras⁺ and K-ras⁻ adenomas differently. However, epidemiological literature on diet, lifestyle and colorectal K-ras mutations is inconsistent.

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Key words: diet; lifestyle; K-ras; colorectal adenomatous polyps; molecular epidemiology

Over the course of time, a set of modifiable risk factors for colorectal cancer and their presumed precursors colorectal adenomas has emerged. These risk factors include excess body weight, physical inactivity, alcohol consumption, smoking, consumption of red and processed meat, low intake of folate, calcium and nonsteroidal antiinflammatory drugs (NSAIDs).^{1,2} However, findings are not entirely consistent.

Mutations in the Kirsten-ras (K-ras) gene are the most common abnormality of oncogenes in human tumours,³ including colorectal adenomas⁴ and carcinomas.^{5,6} The K-ras gene encodes the p21ras protein: a guanine nucleotide binding-protein that plays a role in transmitting growth-stimulatory signals from membrane-bound tyrosine kinases through a group of downstream regulators to the nucleus. When a point mutation occurs in the K-ras gene, the ras protein is locked in its active state leading to a prolonged mitotic signal and thereupon to cell growth and tissue proliferation.⁷

K-ras mutations occur more frequently in adenomas with a villous histology.⁸ Especially glycine-to-valine mutations in codon 12 of the gene may predispose to more aggressive clinical behaviour in colorectal cancer.⁹ It has also been suggested that K-ras mutational status may distinguish 2 groups of familial nonhereditary nonpolyposis colorectal cancer patients whose cancers cur-

rently have unknown genetic origins.¹⁰ The beforementioned pathological and clinical differences may imply that the aetiology of K-ras mutation-positive and mutation-negative adenomas may also differ, also potentially according to specific type of mutation.

Indeed, environmental carcinogens, such as *N*-nitroso compounds that are endogenously formed after red meat consumption or derived from cigarette smoke or processed meat,^{11,12} and polycyclic aromatic hydrocarbons present in cigarette smoke and formed during grilling of meat over direct flame,¹¹ may induce (specific) K-ras mutations.^{13–15} Environmental factors may also select for K-ras mutation-negative lesions as suggested for calcium¹⁶ and heterocyclic amines¹⁷ that are formed during meat cooking at high temperature,^{11,12} for instance by reducing the mutation rate; the latter was shown for *n*-3 polyunsaturated fatty acids in rats.¹⁸ Moreover, environmental factors may affect growth of tumours of K-ras mutation-positive and -negative tumours differently. The NSAID sulindac, for example, selectively modified amplification of cells with a K-ras mutation in codon 12 in rats.¹⁹

In spite of this, the potential environmental influences on the occurrence and persistence of K-ras mutations are largely unknown,³ especially in humans. To date, only one cross-sectional study has evaluated whether the distributions of risk factors differ between patients with K-ras mutation-positive and -negative colorectal adenomas: K-ras mutation-positive adenoma patients were more likely to have a lower intake of total folate and tended to have higher level of vigorous aerobic exercise than did K-ras mutation-negative adenoma patients.²⁰ We conducted a case-control study in the Netherlands to further evaluate whether associations between presumed dietary and lifestyle risk factors and risk of colorectal adenomas depend on K-ras mutational status.

Material and methods

Study population

Cases and controls were recruited among those undergoing endoscopy of the large bowel in the outpatient clinics of 10 hospitals in the Netherlands between June 1997 and June 2000. Medical review boards of all participating hospitals and Wageningen University approved the study protocol. Potential participants were

Abbreviations: A, adenine; C, cytosine; CI, confidence interval; FFQ, food-frequency questionnaire; G, guanine; K-ras, Kirsten-ras; MASA, mutant-allele-specific amplification; NSAID, nonsteroidal antiinflammatory drug; OR, odds ratio; PCR, polymerase chain reaction; T, thymine.

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informed about the study through printed material handed to them, at the time of the endoscopy, by staff, or mail within 3 months thereof. The overall response rate was 54% and varied from 35 to 91% in the various outpatient clinics. To be eligible, participants had to be between 18 and 75 years at the time of endoscopy, be a Caucasian and be able to speak Dutch. They also had to be free of hereditary colorectal cancer syndromes, chronic inflammatory bowel diseases and should not have a history of colorectal cancer or (partial) bowel resection. All participants provided written informed consent. Cases were defined as participants who had at least 1 histologically confirmed colorectal adenoma within 3 years of recruitment ($n = 658$). Controls were never diagnosed with any type of polyps ($n = 709$). Ninety-two percent of the cases and 85% of the controls underwent a full endoscopy (*i.e.* full colonoscopy or sigmoidoscopy combined with X-ray).

Exposure assessment

Participants were requested to fill out a questionnaire on lifestyle and socioeconomic factors, a validated semiquantitative food-frequency questionnaire (FFQ)^{21,22} and a questionnaire on meat consumption and meat preparation methods²³ covering the year preceding study inclusion or bowel complaints.

The FFQ assessed the habitual consumption of 178 foods and covered at least 90% of the population mean intake of the food groups and nutrients of interest in the Netherlands.²¹ For most items, consumption could be indicated per day, month or year. For some foods that are consumed less regularly, consumption frequency could be reported by ticking always/most, often, sometimes or rarely/never. Quantity of consumption could be indicated in prespecified units for most foods, photographs of 2–4 different-sized portions were shown to estimate this for 21 foods, and standard portion sizes were assumed for a few foods. Daily consumption in grams per day was calculated by multiplying consumption frequency per day by portion size. Energy and nutrient intake were calculated using the 1996 computerized version of the Dutch food composition table.²⁴ The questionnaire seems adequate for ranking according to intake of most food groups and nutrients ($r \geq 0.40$ vs. dietary records), although the relative validity for vegetables ($r = 0.38$ for men and 0.31 for women), fish ($r = 0.32$ for men and 0.37 for women), β -carotene ($r = 0.23$ for both sexes), vitamin C for men ($r = 0.37$) and vitamin E for women ($r = 0.35$) was low. The 6- and 12-month reproducibility of the assessment was high for all food groups and nutrients ($r \geq 0.45$).^{21,22}

The additional questionnaire on meat, described in detail elsewhere,²³ assessed consumption frequency and portion sizes of 16 types of meat, gravy and meat preparation methods (*e.g.* height of heat source, addition of water, use of lid). Besides, colour photographs of fried beef patties, pork chops, steak and bacon, which originated from a Swedish questionnaire,²⁵ were included to assess habitual colour of meat surface as proxy for preparation at 225, 200, 175 and 150°C.

Adenoma characteristics and K-ras mutation analysis

We reviewed relevant medical records to obtain information on the indication for endoscopy, polyp history, completeness of colonoscopy and adenoma location, size and number. Besides, the national pathology number of the tumour tissue was recorded to enable retrieval. We obtained tumour tissue from 615 of the 658 eligible adenoma patients (93%) for K-ras mutation analysis. One experienced gastrointestinal pathologist judged the histopathology of all obtained specimens. One sample turned out to be a carcinoma and 6 to be hyperplastic polyps, 15 samples did not contain any polyp tissue, tissue was sparse in 44 and the quality of 1 sample was poor; these 67 samples were excluded.

DNA was extracted from the formalin-fixed paraffin-embedded tissue (10–12 sections, 10 μ m thick) using the PuregeneTM DNA isolation kit (Gentra Systems, Minneapolis, MN). Microdissection was performed by a hematoxylin and eosin-stained 4 μ m section, and only those areas containing at least 60% tumour cells were

used. The tissue was incubated overnight at 55°C in 500 μ l lysis solution containing 0.5 mg/ml Proteinase K (Roche Diagnostics, Mannheim, Germany), followed by 72 hr at 37°C. Proteins were removed by precipitation according to the manufacturer's protocol. DNA was precipitated with 500 μ l 100% isopropanol at 4°C for 30 min. The pellet was washed with 500 μ l 70% ethanol, air-dried, and subsequently, the DNA was rehydrated in 30 μ l DNA hydration solution.

Codons 12 and 13 of the K-ras gene were examined for mutations by direct sequencing in most samples. Polymerase chain reactions (PCRs) were performed in 35 cycles of 30 seconds at 94°C, 45 sec at 55°C and 1 min at 72°C, followed by a final extension step at 72°C for 5 min. The PCR reaction mixture consisted of 100 ng of purified DNA, 20 mmol/l (NH₄)₂SO₄, 75 mmol/l Tris-HCl (pH 9.0), 0.01% Tween, 200 μ mol/l dNTPs, 2.5 mmol/l MgCl₂ and 0.4 μ mol/l of the following primers: ACTCAT-GAAAATGGTCAGAG (3'-primer) and GTACTGGTGGAG-TATTTGATAG (5'-primer). PCR products were checked on an ethidium bromide-stained 2% agarose gel and purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Subsequently, the PCR products were sequenced using ABI PRISM[®] BigDyeTM Terminators v3.0 (Applied Biosystems, Foster City, CA) and cycle sequencing with AmpliTaq[®] DNA polymerase, FS (Applied Biosystems). Sequencing was performed in both directions using the same primers. Samples were analyzed on an ABI PRISM[®] 3700 DNA Analyzer (Applied Biosystems). Forty-two samples were analyzed using mutant-allele-specific amplification (MASA)-PCR as described previously,²⁶ 12 of which were mutation-positive. The direct sequencing method and MASA-PCR methods allowed detection of the presence of 5% and 10–15% mutant DNA, respectively. Additionally, we performed sequence analyses on a number of samples ($n = 8$), each with a mutation in codon 12 or 13 according to MASA. In all samples, the same mutation was found.

K-ras mutation analysis was successful in 534 of the 548 available samples, yielding a study population of 81 cases with K-ras mutation-positive adenomas, 453 cases with K-ras mutation-negative adenomas and 709 controls.

Statistical analysis

In the analyses, we evaluated factors that are thought to be risk factors for colorectal adenomas and carcinomas,^{1,2} hypothesized to be related to K-ras mutational status^{3,13,20,26–29} and factors that were previously associated with colorectal adenomas in our study population.^{30,31}

The distributions of demographic and tumour characteristics and presumed dietary and lifestyle risk factors were examined visually and numerically for cases according to K-ras mutational status and controls. All nutrients bar alcohol were adjusted for total energy intake using the residual method.³² Continuous exposure variables were divided into tertiles based on the distribution among controls. The distributions of risk factors among K-ras mutation-positive and -negative cases were compared to evaluate etiologic heterogeneity using multinomial logistic regression (case–case analyses). Simultaneously, odds ratios (ORs) and 95% confidence intervals (CIs) were estimated for K-ras mutation-positive and -negative adenomas separately vs. controls to evaluate whether risk factors were positively or inversely associated with the disease (case–control analyses).

Separate models, which always included age (continuous), gender and total energy intake (kJ/day, continuous), were fitted for each factor under investigation. One of the other examined factors, such as family history of colorectal cancer, indication for endoscopy (bowel-complaint related yes/no), education level or hormone replacement therapy (set to 0 for men) was added in turn to evaluate potential confounding. Red meat changed the parameter estimate for monounsaturated fat by more than 10% and was therefore added to the model for this factor. None of the other fac-

tors changed specific parameter estimates by more than 10%, and they were therefore not included in the final models.

An ordinal score value based on the median value within each tertile among controls was used to test for trend across tertiles. We also evaluated whether the distribution of risk factors over tertiles or exposure categories differed for patients having codon 12 transitions and codon 12 transversion mutations by Fisher's exact test, whereas we visually examined the distribution of codon 13 mutations.

As sensitivity analyses, we evaluated whether the parameter estimates changed significantly when we excluded participants who indicated to have ever changed their diet, who did not undergo a full endoscopy, cases who were previously diagnosed with an adenoma, had multiple adenomas, advanced adenomas [≥ 1 cm or (tubulo)villous structure] or no adenomas in the distal colon and samples that were analyzed by MASA-PCR in turn. Furthermore, we evaluated whether the associations were driven by potential differential consumption patterns of men and women by using gender-specific tertiles. We also applied quartiles, instead of tertiles, in the analyses.

All reported p -values are two-sided and regarded as statistically significant if less than 0.05. We used Statistical Analysis Software (SAS version 9.1, SAS Institute, Cary, NC) for all analyses.

Results

Table I shows the characteristics of *K-ras* mutation-positive (*K-ras*⁺) and -negative (*K-ras*⁻) cases, cases in whose tissue *K-ras* mutational status could not be assessed and controls. The entire case group was slightly older and consisted of relatively more men and smokers. A smaller proportion of cases than controls underwent endoscopy for bowel complaints. The case group had a higher intake of total energy, meat, fruits, vegetables and alcohol compared with the control group. The characteristics of cases in whose tissue *K-ras* mutational status could not be assessed were largely comparable to the characteristics of *K-ras*⁻ cases, although they resembled *K-ras*⁺ cases more with respect to gender and consumption of fruits. Fifteen percent of the cases in which we could assess *K-ras* mutational status harboured mutations in the gene. There were fewer smokers among *K-ras*⁺ cases than among *K-ras*⁻ cases. On average, *K-ras*⁺ cases had a higher total energy intake, and consumed more fruit compared with *K-ras*⁻ cases. A smaller proportion of *K-ras*⁺ cases had adenomas before, but a larger proportion was diagnosed with multiple adenomas compared with *K-ras*⁻ cases. The majority of *K-ras*⁺ adenomas had villous or tubulovillous histology and were large, whereas the majority of *K-ras*⁻ adenomas were tubular and small. Relatively more *K-ras*⁻ adenomas occurred in the distal colon.

Among the 81 specimens with *K-ras* mutations, 66 carried mutations in codon 12 exclusively (62 monoallelic, 4 biallelic), 14 carried a monoallelic mutation in codon 13 exclusively, and one carried both a biallelic codon 12 mutation and a monoallelic codon 13 mutation. Transversion from *K-ras* codon 12 GGT to GTT (Gly→Val) was the most common mutation ($n = 29$), followed by transition to GAT (→Ala, $n = 23$). Other mutations in codon 12 were to TGT (→Cys, $n = 6$), AGT (→Ser, $n = 6$), CGT (→Arg, $n = 5$), and GCT (→Ala, $n = 3$). In codon 13, only transition mutations from GGC to GAC (Gly→Asp) occurred.

Table II presents case-case comparisons according to potential modifiable risk factors. Cases with *K-ras*⁺ adenomas were more likely to have a lower intake of monounsaturated fat and a higher intake of vitamin B2 than cases with *K-ras*⁻ adenomas. The distributions of total dietary and polyunsaturated fat, calcium and red meat, and less strikingly, of protein, dairy products, tea and (duration of) smoking also appeared to differ between *K-ras*⁺ and *K-ras*⁻ cases, although not statistically significant. Dairy products, calcium and protein were strongly correlated with the intake of vitamin B2 (Spearman's $r \geq 0.60$). The inverse association with dairy products was still visible when we added vitamin B2 or calcium to the model, albeit the case-case comparison hardly showed

differences anymore (highest vs. lowest tertile: OR = 0.74 and p trend = 0.58; OR = 1.09 and p trend = 0.82, respectively). Nonetheless, when we added dairy products, calcium or protein to the model for vitamin B2, the case-case comparison still indicated differences between *K-ras*⁺ and *K-ras*⁻ adenomas (OR = 2.37 and p trend = 0.075; OR = 1.67 and p trend = 0.20; OR = 1.78 and p trend = 0.083, respectively). This was also the case when we added dairy products (OR = 1.70 and p trend = 0.23) or protein (OR = 1.64 and p trend = 0.18) to the model on calcium. However, addition of vitamin B2 decreased the strength of the case-case association considerably (OR = 1.20 and p trend = 0.73). Similarly, the potential differential association with protein disappeared when we added vitamin B2 to the model (OR = 1.13 and p trend = 0.71). We did not find indications for differential associations according to meat preparation methods (estimates not shown). No indications for differential associations with *K-ras*⁺ and *K-ras*⁻ adenomas could be detected for the other examined factors, including specific subgroups of fruits and vegetables (estimates not shown) and coffee consumption (median consumption: 4 cups/day, 25th percentile = 3 cups/day, 75th percentile = 6 cups/day, equal for *K-ras*⁺ and *K-ras*⁻ cases). When we compared regular consumption of coffee (≥ 2 glasses/day) with low/intermediate consumption (< 2 glasses/day), also no difference in distribution among *K-ras*⁺ and *K-ras*⁻ adenoma patients could be detected (OR = 1.41, 95% CI = 0.53–3.73).

Differences in risk of *K-ras*⁺ and *K-ras*⁻ adenomas were only statistically significant for intake of monounsaturated fat and vitamin B2. The case-control analyses presented in Table III illustrate why these differences in risk arose; the table also presents case-control analyses for other factors that were possibly differently associated with risk of *K-ras*⁺ and *K-ras*⁻ adenomas. Consumption of red meat was positively associated with *K-ras*⁺ adenomas, but not with *K-ras*⁻ adenomas. The intake of total dietary, polyunsaturated and monounsaturated fat tended to be inversely associated with risk of *K-ras*⁺ adenomas. However, especially intake of monounsaturated fat was positively associated with risk of *K-ras*⁻ adenomas. Intake of vitamin B2 was somewhat positively, but not statistically significantly, associated with *K-ras*⁺ adenomas. However, vitamin B2 was inversely associated with *K-ras*⁻ adenomas. Similar patterns were observed for dairy products, calcium and protein. No association between tea consumption and *K-ras*⁺ adenomas could be detected, but tea was inversely, although not statistically significantly, associated with risk of *K-ras*⁻ adenomas. Smoking, especially its duration, was relatively strongly positively associated with risk of *K-ras*⁻ adenomas, but only weakly so with *K-ras*⁺ adenomas.

The aforementioned findings were supported by the sensitivity analyses. The analyses in which gender-specific tertiles were used and with restriction to those who underwent full endoscopy suggested that high physical activity tended to decrease the risk of *K-ras*⁻ adenomas, but somewhat increase the risk of *K-ras*⁺ adenomas, albeit this was not statistically significant.

Strata according to specific mutations were very small. However, remarkably, none or a very low number of patients who developed an adenoma harbouring a codon 13 mutation were classified in the lowest tertile of consumption or intake of fruit and vegetables, folate and less strikingly, of β -carotene and vitamin C (results not shown). It was also remarkable that a higher proportion of patients with codon 12 transversion mutations were classified in the highest tertile of fruits, green leafy vegetables and folate compared with patients with codon 12 transition mutations ($p < 0.05$; results not shown).

Discussion

We observed that intake of vitamin B2 and monounsaturated fat were differently associated with risk of *K-ras* mutation-positive and -negative adenomas. Similar, not statistically significant, differences in risk were observed for red meat, dairy products, total

TABLE 1 – DESCRIPTION OF THE STUDY POPULATION

Characteristic	Cases ¹			Controls ¹ (n = 709)
	K-ras mutation-positive adenomas (K-ras ⁺) (n = 81)	K-ras mutation-negative adenomas (K-ras ⁻) (n = 453)	Tissue unavailable or assessment unsuccessful ² (n = 124)	
Demographics and lifestyle factors				
Age (years), mean ± SD	60.3 ± 9.6	58.7 ± 10.1	58.8 ± 10.1	51.5 ± 13.6
Female, n (%)	39 (48.2%)	203 (44.8%)	61 (49.2%)	437 (61.6%)
BMI, kg/m ² , mean ± SD	26.1 ± 4.2	26.1 ± 3.8	26.7 ± 4.1	25.5 ± 4.1
Low education level, n (%) ³	30 (44.1%)	142 (35.0%)	45 (38.1%)	215 (33.0%)
Ever smoking, n (%)	49 (61.3%)	310 (68.7%)	79 (64.2%)	389 (55.2%)
Duration of smoking among smokers (years), median (25th; 75th percentile)	25 (20; 41.5)	30 (20; 40)	30 (20; 39)	22.5 (13; 32)
High physical activity ⁴ , n (%)	24 (32.9%)	124 (29.1%)	32 (27.8%)	228 (33.5%)
Medical history, n (%)				
Family history of colorectal cancer	18 (22.2%)	102 (22.5%)	30 (24.2%)	136 (19.2%)
More than 12 times NSAIDs per year	23 (28.4%)	117 (25.8%)	30 (24.2%)	207 (29.2%)
Bowel complaints or defecation problems as indication of endoscopy	36 (46.2%)	241 (54.2%)	49 (39.8%)	525 (77.0%)
Dietary factors, median (25th; 75th percentile)				
Total energy intake (kJ/day)	8,897 (7,224; 9,717)	8,336 (7,035; 10,177)	8,224 (6,984; 9,748)	8,112 (6,758; 9,935)
Protein (g/day)	77.5 (69.0; 94.4)	76.2 (64.3; 89.9)	78.4 (65.6; 91.0)	74.6 (62.2; 90.0)
Carbohydrates (g/day)	227.9 (183.6; 269.9)	216.1 (177.3; 265.0)	216.2 (179.5; 267.7)	223.1 (180.3; 267.7)
Fat (g/day)	82.5 (63.8; 94.7)	79.4 (62.4; 98.4)	79.8 (63.2; 94.7)	75.1 (58.4; 97.2)
Fruits (g/day) ⁵	152.4 (86.5; 246.8)	125.9 (71.1; 248.9)	169.9 (74.0; 248.9)	124.7 (72.5; 243.9)
Vegetables (g/day) ⁶	118.9 (95.7; 161.4)	115.7 (90.4; 140.8)	122.0 (95.7; 150.4)	109.3 (83.7; 139.2)
Dietary fibre (g/day)	23.2 (20.0; 28.5)	23.7 (19.2; 28.3)	23.8 (19.4; 27.9)	22.6 (18.8; 27.2)
Meat (g/day)	108.8 (69.7; 134.3)	107.2 (70.1; 144.0)	106.8 (70.6; 136.7)	101.7 (63.1; 133.0)
Alcohol (g/day)	11.6 (1.7; 21.8)	8.7 (1.0; 24.1)	8.1 (0.3; 15.4)	4.2 (0.3; 15.4)
Adenoma, n (%)	12 (15.0%)	100 (22.1%)	37 (30.1%)	0 (0%)
Adenoma history				
Histopathology ⁷				
Tubular	33 (40.7%)	369 (82.2%)	84 (78.5%)	—
Tubulovillous	22 (27.2%)	56 (12.5%)	18 (16.8%)	—
Villous	26 (32.1%)	24 (5.4%)	5 (4.7%)	—
Location⁸				
Rectum	14 (25.9%)	40 (13.8%)	16 (22.5%)	—
Distal colon	31 (57.4%)	205 (70.7%)	44 (62.0%)	—
Proximal colon	9 (16.7%)	45 (15.5%)	11 (15.5%)	—
Large (≥ 1 cm)	48 (64.0%)	160 (37.0%)	39 (34.2%)	—
Multiple adenomas	46 (59.0%)	185 (42.0%)	53 (42.7%)	—

¹Because of missing values, not all numbers sum up to the column totals. ²No tissue obtained from 43, 67 samples did not contain (sufficient) polyp tissue, K-ras assessment unsuccessful for 14; see methods section. ³Primary school or lower vocational training only. ⁴Individuals classified in the highest tertile of a continuous activity score (based on distribution among controls). ⁵Excluding juices and apple sauce. ⁶Excluding potatoes. ⁷As assessed by the study pathologist for tissue that was available and obtained from the status information when the tumour block was not present or could not be judged, which was the case for most adenomas in which K-ras mutation status could not be determined. Histopathology of the examined polyp was not evaluated for 4 K-ras⁺ adenomas and 17 adenomas in which K-ras mutation status could not be assessed. ⁸Recorded from status information, in which the location of the retrieved adenoma was not always possible to retrieve, in particular when multiple polyps were diagnosed.

TABLE II - CASE-CASE COMPARISONS: ODDS RATIOS OF K-ras MUTATION-POSITIVE (K-ras⁺) VERSUS -NEGATIVE ADENOMAS (K-ras⁻) ACCORDING TO TERTILES OR CATEGORIES OF POTENTIAL MODIFIABLE RISK FACTORS^{1,2}

Characteristic	Cut-off points for classification of exposure		Number of cases, low/intermediate/high		Intermediate versus low exposure		High versus low exposure		p for trend ²
	Low	High	K-ras ⁺	K-ras ⁻	OR (95% CI)	OR (95% CI)	OR (95% CI)		
Foods									
Dairy products									
Total meat	<238.2 g/day	>474.6 g/day	25/27/29	173/148/132	1.24 (0.69–2.25)	1.52 (0.83–2.81)	1.52 (0.83–2.81)	0.17	
Red meat ³	<74.6 g/day	>121.7 g/day	22/28/31	125/147/181	1.15 (0.62–2.13)	1.10 (0.56–2.15)	1.10 (0.56–2.15)	0.78	
Processed meat	<38.2 g/day	>70.5 g/day	22/21/38	138/156/159	0.92 (0.48–1.77)	1.69 (0.92–3.11)	1.69 (0.92–3.11)	0.063	
Poultry	<18.5 g/day	>39.9 g/day	27/31/23	144/145/164	1.16 (0.65–2.06)	0.78 (0.40–1.53)	0.78 (0.40–1.53)	0.41	
Fish	<5.1 g/day	>13.7 g/day	26/30/25	128/165/160	0.93 (0.52–1.67)	0.81 (0.44–1.49)	0.81 (0.44–1.49)	0.51	
Fruits	<4.28 g/day	>14.5 g/day	31/24/26	138/170/145	0.62 (0.35–1.11)	0.76 (0.42–1.35)	0.76 (0.42–1.35)	0.38	
Vegetables	<105.5 g/day	>230.8 g/day	21/31/29	149/144/160	1.45 (0.79–2.68)	1.21 (0.65–2.26)	1.21 (0.65–2.26)	0.77	
Macronutrients	<91.9 g/day	>128.5 g/day	18/29/33	119/162/167	1.17 (0.62–2.22)	1.27 (0.68–2.39)	1.27 (0.68–2.39)	0.48	
Total energy	<7173.8 kJ/day	>9227.9 kJ/day	20/28/33	127/162/164	1.20 (0.64–2.25)	1.57 (0.81–3.05)	1.57 (0.81–3.05)	0.28	
Carbohydrates	<212.3 g/day	>241.6 g/day	24/35/22	161/131/161	1.84 (1.03–3.31)	0.97 (0.48–1.97)	0.97 (0.48–1.97)	0.99	
Total dietary fat	<71.2 g/day	>84.5 g/day	29/19/33	108/138/207	0.48 (0.24–0.94)	0.50 (0.23–1.06)	0.50 (0.23–1.06)	0.11	
Polysaturated fat	<13.1 g/day	>16.5 g/day	32/22/27	119/150/184	0.53 (0.29–0.99)	0.52 (0.27–0.98)	0.52 (0.27–0.98)	0.062	
Monounsaturated fat	<26.9 g/day	>32.3 g/day	30/18/33	110/138/205	0.40 (0.20–0.80) ^{4a}	0.42 (0.20–0.90) ^{4b}	0.42 (0.20–0.90) ^{4b}	0.044 ^{4c}	
Saturated fat	<29.6 g/day	>35.7 g/day	20/32/29	108/143/202	1.17 (0.62–2.20)	0.73 (0.35–1.49)	0.73 (0.35–1.49)	0.29	
Protein	<71.0 g/day	>81.6 g/day	21/23/37	133/141/179	1.12 (0.58–2.15)	1.57 (0.80–3.11)	1.57 (0.80–3.11)	0.16	
Animal protein	<44.0 g/day	>54.1 g/day	21/22/38	130/152/171	0.91 (0.48–1.76)	1.44 (0.78–2.65)	1.44 (0.78–2.65)	0.19	
Micronutrients									
Dietary fibre	<20.8 g/day	>24.8 g/day	23/23/35	128/138/187	0.93 (0.50–1.74)	1.06 (0.58–1.92)	1.06 (0.58–1.92)	0.81	
Vitamin B2	<1.35 mg/day	>1.75 mg/day	18/27/36	126/193/134	0.97 (0.51–1.87)	1.88 (0.99–3.55)	1.88 (0.99–3.55)	0.031	
Vitamin B6	<1.44 mg/day	>1.70 mg/day	22/26/33	109/147/197	0.89 (0.47–1.69)	0.88 (0.45–1.73)	0.88 (0.45–1.73)	0.46	
Folate	<170.8 µg/day	>202.2 µg/day	17/27/37	106/146/201	1.16 (0.60–2.26)	1.20 (0.63–2.32)	1.20 (0.63–2.32)	0.60	
Vitamin B12	<3.62 mg/day	>4.83 mg/day	17/29/35	127/136/190	1.64 (0.85–3.16)	1.43 (0.74–2.76)	1.43 (0.74–2.76)	0.43	
Calcium	<916.0 mg/day	>1208.4 mg/day	18/31/32	145/171/137	1.39 (0.74–2.62)	1.80 (0.96–3.38)	1.80 (0.96–3.38)	0.072	
Lifestyle factors									
Alcohol	<1 glass/week	>10 glasses/week	20/26/34	131/153/170	1.12 (0.60–2.10)	1.38 (0.74–2.58)	1.38 (0.74–2.58)	0.29	
Coffee	<3 cups/day	>5 cups/day	21/36/24	157/140/156	1.95 (1.08–3.52)	1.26 (0.66–2.42)	1.26 (0.66–2.42)	0.46	
Tea	<1 cups/day	>3 cups/day	29/25/27	197/138/118	1.18 (0.66–2.13)	1.46 (0.81–2.63)	1.46 (0.81–2.63)	0.21	
Smoking ⁵	Never smokers	Current smokers	31/32/17	141/177/133	0.83 (0.48–1.45)	0.62 (0.32–1.20)	0.62 (0.32–1.20)	0.16	
Duration of smoking	Never	>22.5 years	31/20/30	141/115/197	0.86 (0.46–1.61)	0.70 (0.40–1.23)	0.70 (0.40–1.23)	0.21	
Physical activity ⁶	Low	High	25/24/24	178/124/124	1.38 (0.75–2.54)	1.38 (0.75–2.54)	1.38 (0.75–2.54)	0.18	
Body mass index	<23.5 kg/m ²	>26.7 kg/m ²	23/25/33	124/158/163	0.87 (0.47–1.63)	1.10 (0.61–1.97)	1.10 (0.61–1.97)	0.66	
Regular NSAID use	<12/year	>12/year	58/-/23	336/-/117	–	1.11 (0.65–1.88)	1.11 (0.65–1.88)	–	

¹Adjusted for sex, age and total energy. All nutrients, except alcohol, were adjusted for energy by the residual method.²The case-control comparisons for factors for which the p value for trend was <0.25 and showed an odds ratio ≥1.4 or equivalently, ≤0.7 are presented in Table III.³Does not include organ meat.⁴Also adjusted for red meat. Without this factor, the estimates were a: 0.46 (0.24–0.90); b: 0.53 (0.26–1.10) and c: 0.13.⁵Intermediate category consists of former smokers.⁶As assessed by a continuous score that was subdivided into tertiles.

TABLE III – CASE-CONTROL COMPARISONS: ODDS RATIOS AND 95% CONFIDENCE INTERVALS FOR K-ras MUTATION-POSITIVE ADENOMAS (K-ras⁺) AND -NEGATIVE ADENOMAS (K-ras⁻) ACCORDING TO SELECTED MODIFIABLE RISK FACTORS¹

Characteristic	N			Case-control comparisons			
	K- <i>ras</i> ⁺	K- <i>ras</i> [−]	Controls	K- <i>ras</i> ⁺ versus controls		K- <i>ras</i> [−] versus controls	
				OR (95% CI)	P trend	OR (95% CI)	P trend
Foods							
Dairy products, excluding cheese and butter (g/day)							
≤238.2	25	173	237	1 (Ref)		1 (Ref)	
>238.2 to 474.6	27	148	235	0.89 (0.50–1.60)		0.72 (0.53–0.97)	
>474.6	29	132	237	0.94 (0.51–1.71)	0.85	0.61 (0.45–0.85)	0.028
Red meat (g/day) ²							
≤38.2	22	138	237	1 (Ref)		1 (Ref)	
>38.2 to 70.5	21	156	235	1.05 (0.55–2.00)		1.14 (0.83–1.56)	
>70.5	38	159	237	1.70 (0.94–3.09)	0.061	1.00 (0.73–1.39)	0.98
Macronutrients							
Total dietary fat (g/day, energy-adjusted)							
≤71.2	29	108	237	1 (Ref)		1 (Ref)	
>71.2 to 84.5	19	138	235	0.55 (0.28–1.06)		1.15 (0.82–1.61)	
>84.5	33	207	237	0.62 (0.30–1.32)	0.30	1.26 (0.84–1.88)	0.29
Polyunsaturated fat (g/day, energy-adjusted)							
≤13.1	32	119	236	1 (Ref)		1 (Ref)	
>13.1 to 16.5	22	150	236	0.62 (0.34–1.13)		1.16 (0.84–1.60)	
>16.5	27	184	237	0.58 (0.31–1.10)	0.11	1.13 (0.80–1.59)	0.56
Monounsaturated fat (g/day, energy-adjusted) ³							
≤26.8	30	110	237	1 (Ref)		1 (Ref)	
>26.8 to 32.3	18	138	236	0.51 (0.25–1.00) ^{3a}		1.27 (0.90–1.78) ^{3d}	
>32.3	33	205	236	0.67 (0.32–1.40) ^{3b}	0.37 ^{3c}	1.57 (1.06–2.34) ^{3e}	0.029 ^{3f}
Protein (g/day, energy-adjusted)							
≤71.0	21	133	236	1 (Ref)		1 (Ref)	
>71.0 to 81.6	23	141	236	0.85 (0.44–1.62)		0.76 (0.55–1.05)	
>81.6	37	179	237	1.10 (0.56–2.15)	0.70	0.70 (0.49–1.00)	0.054
Micronutrients							
Calcium (mg/day, energy-adjusted)							
≤916.0	18	145	237	1 (Ref)		1 (Ref)	
>916.0 to 1208.4	31	171	235	1.37 (0.73–2.57)		0.99 (0.73–1.34)	
>1208.4	32	137	237	1.27 (0.68–2.36)	0.50	0.71 (0.51–0.97)	0.028
Vitamin B2 (mg/day, energy-adjusted)							
≤1.35	18	126	237	1 (Ref)		1 (Ref)	
>1.35 to 1.75	27	193	235	1.10 (0.58–2.11)		1.13 (0.83–1.55)	
>1.75	36	134	237	1.31 (0.71–2.44)	0.33	0.70 (0.50–0.97)	0.020
Lifestyle factors							
Tea (cups/day)							
<1	29	197	290	1 (Ref)		1 (Ref)	
1–<3	25	138	208	1.02 (0.57–1.82)		0.86 (0.64–1.16)	
≥3	27	118	211	1.08 (0.60–1.94)	0.79	0.74 (0.54–1.02)	0.065
Smoking							
Never	31	141	316	1 (Ref)		1 (Ref)	
Former	32	177	216	1.20 (0.70–2.08)		1.45 (1.08–1.96)	
Current	17	133	173	1.27 (0.66–2.42)	0.44	2.04 (1.47–2.83)	<0.0001
Duration of smoking							
Never	31	141	316	1 (Ref)		1 (Ref)	
0–22.5 years	20	115	203	1.30 (0.70–2.42)		1.52 (1.10–2.11)	
≥22.5 years	30	197	190	1.25 (0.71–2.18)	0.45	1.79 (1.33–2.42)	0.0002

¹Only factors for which the p-value for trend was <0.25 and showed an odds ratio ≥1.4 or ≤0.7 in the case-case analyses (see Table II) are presented in this table. Odds ratios are adjusted for sex, age and total energy.²Does not include organ meat.³Also adjusted for red meat. Without this factor, the estimates were a: 0.58 (0.30–1.12); b: 0.81 (0.40–1.66); c: 0.68; d: 1.26 (0.90–1.76); e: 1.53 (1.04–2.25) and f: 0.034.

dietary and polyunsaturated fat, protein, calcium, tea and (duration of) smoking. These differences in risk mainly arose because inverse associations with vitamin B2, dairy products, calcium and possibly protein and tea were confined to K-ras mutation-negative adenomas. Intake of total dietary, monounsaturated and polyunsaturated fat tended to be inversely associated with risk of K-ras mutation-positive adenomas, but not, or somewhat positively, with risk of K-ras mutation-negative adenomas. Smoking and its duration were more clearly positively associated with K-ras mutation-negative than with mutation-positive adenomas. A positive association with red meat was distinctly found for K-ras mutation-positive adenomas. Additionally, we found hints that the distribution of specific K-ras mutations may vary according to intake of folate and consumption of fruits and vegetables.

In the present analyses, we mainly focused on the comparisons of the distributions of risk factors among K-ras mutation-positive and -negative cases. Such comparisons are unlikely to be affected by information bias; reporting of diet and lifestyle is implausible to be related to mutational status. We cannot completely rule out selection bias that occurs when factors determining tumour block availability or successful K-ras assessment are also associated with exposure factors of interest and K-ras mutation status.^{33,34} However, its impact is likely to be minor; the distribution of risk factors and adenoma characteristics of the tumours in which mutational status could not be assessed appeared to be largely comparable to the weighted average of the distributions among K-ras mutation-positive and -negative adenoma cases. Risk of selection and information bias remains a weak point regarding the case-

control comparisons, although it is reassuring that excluding participants who indicated to have changed their diet did not affect the results.

In our study, 15% of the adenomas harboured a K-*ras* mutation. This percentage is in the same range of magnitude as the 18% observed in a cross-sectional study on K-*ras* mutations in colorectal adenomas.²⁰ Reported mutation rates are generally higher, e.g. a compilation of 12 reports on 646 adenomas, which did not include the cross-sectional study, reported a rate of 37%.⁴ The latter estimate may be higher because it was partly based upon adenomas diagnosed in cancer patients, whereas the cross-sectional study and our study were based upon a cancer-free endoscopy population. Alternatively, we may have underestimated the mutation rate because we studied 1 adenoma per patient and could not always study the entire adenoma, whereas mutational patterns may differ between³⁵ and within adenomas of the same patient.³⁶ Besides, we did not evaluate the presence of K-*ras* codon 61 mutations, but these are rare.³⁷ We expect that bias due to the use of 2 different methods of K-*ras* assessment is small, because exclusion of samples analysed with MASA-PCR did not change the results. More substantial errors could have arisen through the multiple comparisons made and the relatively small sample size of K-*ras* mutation-positive cases. Consequently, some detected differences may be due to chance, and we may not have had sufficient power to detect existing associations.

Only one study evaluated associations between presumed risk factors and K-*ras* mutation-positive and -negative adenomas before.²⁰ In that cross-sectional study, K-*ras* mutation-positive adenoma cases ($n = 120$) were less likely to have a high folate intake, and possibly less likely to participate in vigorous aerobic exercise than K-*ras* mutation-negative adenoma cases ($n = 558$).²⁰ Differences in study population or design, and the relatively small sample size of K-*ras* mutation-positive samples in both studies may partly explain why we observed associations with different factors. Levels of intake may not have been high enough to allow detection of an association with calcium in the cross-sectional study²⁰ and with folate in our study; indeed, the cross-sectional study observed an association with total but not with dietary folate.²⁰ Even so, it is unlikely that the aforementioned factors account for all discrepancies, as epidemiological studies on environmental risk factors and risk of K-*ras* mutation-positive and -negative colorectal cancers also yielded inconsistent results.^{26–29,38–43}

The inverse association with calcium, for instance, was restricted to K-*ras* mutation-negative adenomas in our study, which is in keeping with a study in which calcium-supplemented rats did not develop K-*ras* mutation-negative tumours.¹⁶ However, it was restricted to mutation-positive colorectal cancers in another study.⁴² Two colorectal cancer studies^{26,29} could not detect any such differential association, although in one of them codon 12 mutations were more likely to be observed than codon 13 mutations when calcium intake was high.²⁶ Part of these differences may be attributed to differences in exposure to compounds associated with the intake of calcium or dairy products, such as vitamin B2 and organochlorines.⁴⁴ Indeed, the association with vitamin B2, a cofactor in folate metabolism,³⁰ tended to be stronger than the association with calcium in our study, and organochlorines, that none of the aforementioned studies assessed, were associated with K-*ras* mutations in other tumours.⁴⁴

Inconsistent findings were also observed regarding other factors, including consumption of red meat,^{26,28,29,38,43} and intake of

fat^{26,29,40,42} and folate.^{29,39,42} Other factors have been studied less frequently,^{27–29,41,42,45} and coffee and tea, whose many constituents exert strong effects upon a wide variety of physiological, cellular and molecular systems,⁴⁶ have not been studied in relation to K-*ras* mutations in adenomas before. Patients with K-*ras* mutation-positive exocrine pancreatic cancer were more likely to be in the upper category of coffee consumption than patients with K-*ras* mutation-negative exocrine pancreatic cancer,⁴⁶ but we could not confirm this regarding colorectal adenomas. Whereas *in vivo* and *in vitro* studies indicate that specific exposures are associated with specific K-*ras* mutations,^{11–15,17,18,44} the general picture regarding associations between exposure and specific K-*ras* mutations obtained from observational studies is also not clear.^{20,26,29,38–40,45}

Besides small sample sizes, the inconsistent findings of epidemiological studies on K-*ras* mutations in colorectal tumours may be partly due to the use of questionnaires to assess exposure. Such an assessment may not be sensitive enough to detect associations; assessment of specific agents hypothesized to affect K-*ras* mutations, including N-nitroso compounds,^{11,12,14} polycyclic aromatic hydrocarbons,¹³ heterocyclic amines,¹⁷ n-3 polyunsaturated fatty acids¹⁸ and organochlorine compounds⁴⁴ in body fluids or tissue samples may be more appropriate. Besides, associations may be diluted because K-*ras* mutations by themselves may not be the best to distinguish unique aetiological pathways,⁴⁷ and some compounds could also be related to mutations in oncogenes, tumour suppressor genes or DNA repair genes in K-*ras* mutation-negative adenomas. On top of this, K-*ras* mutations occur more frequently in strong dysplastic areas,⁴⁸ which is also reflected in the heterogeneous K-*ras* mutation pattern within an adenoma³⁶ and the lack of concordance between K-*ras* mutational status in multiple adenomas of the same patient.³⁵ Thus, associations of diet and lifestyle with K-*ras* mutational status may be hard to confirm in epidemiological studies.

In conclusion, our findings add to the body of evidence that risk factors may play a different role in the aetiology of K-*ras* mutation-positive and mutation-negative colorectal adenomas. Nevertheless, findings on dietary and lifestyle factors and K-*ras* mutations in colorectal tumours remain highly inconsistent in the literature.

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