Decrease in Specific Micronutrient Intake in Colorectal Cancer Patients with Tumors Presenting Ki-*ras* Mutation

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Abstract. *Background: The diversity of the Mediterranean diet and the heterogeneity of acquired genetic alterations in colorectal cancer (CRC) led us to examine the possible association between dietary factors and mutations, such as Ki-ras mutations, in genes implicated in the pathogenesis of these neoplasms. Patients and Methods: The study was based on 246 cases and 296 controls. For the molecular study only 117 patients with Ki-ras tumor expression were included. Dietary patterns were assessed using a semi-quantitative food frequency questionnaire. Results: Patients with Ki-ras mutations in codon 12 (K12) consumed significantly less vitamin A (p=0.02), B1 (p=0.01), D (p=0.02) and iron (p= 0.03) than controls, whereas patients without these mutations had similar intakes of these nutrients to controls. The consumption of fiber, folate, vitamin E and potassium was lower in the two subgroups of patients (K12-positive or -negative) than in controls. Mutation in codon 13 was not associated with any nutrient deficit. Conclusion: These results support previous findings that certain micronutrients protect against colorectal neoplasia and emphasize the importance of considering the different molecular forms of CRC as etiologically distinct diseases.*

Although it is well established that cancer in humans occurs as a process involving multiple and variable events and stages, little is known about how these events relate to variability in outcome. Variability in colorectal tumors is reflected in the diversity of genetic alterations observed in tumors themselves, as well as by differences in age at diagnosis and in age- and site-specific risk factors (1-3). Specific exposures that constitute risk factors for colon cancer may contribute to the heterogeneity of acquired genetic alterations observed in colon tumors. Therefore, it is reasonable to hypothesize that diet and other lifestyle and environmental factors are associated with some mutations but not with others. Mutations in the Ki-*ras* gene were among the first to be linked to the pathogenesis of colon cancer (4). Ki-*ras* mutations are thought to be early events in colon cancer pathogenesis because they are observed in both adenomas and carcinomas (5-7). Most studies estimate that 30-50% of adenomas and carcinomas have Ki-*ras* mutations; almost 90% of Ki-*ras* mutations in colorectal tumors occur in codons 12 and 13 (7-9).

Data to support specific associations between diet and genetic mutations in tumors are limited. In one case-control study of 108 sporadic colorectal cancers (CRC), low levels of calcium and high levels of monounsaturated fat were associated with increased odds of having Ki-*ras*-positive tumors (10). Another study of adenomas observed that high levels of dietary plus supplemental folate were associated with decreased odds of having Ki-*ras* adenomas; dietary folate without supplements was not associated with Ki- *ras*-positive adenomas (11). In an extensive study by Slattery *et al.*, folate and vitamin B6 were found to protect against Ki-*ras* mutations (12). However, since previous studies of diet and Ki-*ras* mutations in colon tumors have included few subjects, it has not been possible to evaluate dietary associations with specific types of Ki-*ras* mutations. The

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study by Martinez *et al.* (11) did evaluate specific types of mutations with dietary factors and found no significant differences in dietary associations with specific types of mutations.

In this study we used data collected as part of a case-control study of sporadic colorectal cancer risk (13, 14) in order to evaluate associations between diet and Ki-*ras* mutations.

Patients and Methods

Study population. Patients were recruited through the University of Barcelona's Hospital Clinic, a 900-bed institution with about 33,000 admissions per year and which serves a source population of approximately 600,000 people within Catalonia, Spain. It is both a teaching institution and tertiary care facility. From 1996-1997, 275 consecutive sporadic CRC patients undergoing surgery were identified through the Clinic's two surgical services as potential subjects for a hospital-based case-control study of CRC. Patients, who were ≥40 years old and <90 years, were invited to participate in the study. Eighteen subjects refused to participate, leaving a final total of 247 enrolled Caucasian subjects (131 men and 116 women). A consecutive case-series subset of 117 patients (originally 120, but three patients were older than 90) within one surgical service also provided tissue specimens (uninvolved colon and tumor) for the molecular study. The remainder of the CRC group only provided blood samples. Primary tumors were surgically dissected and immediately frozen at -80°C. Patients gave their signed informed consent and all procedures were approved by the Ethical Research Board of the Hospital Clinic. A questionnaire was administered to each patient by an interviewer; the questionnaire elicited demographic information, data on occupation and smoking habits and personal medical history. The primary measure of smoking was the pack year unit (PY: number of packets of 20 cigarettes per day x number of years). Clinical and pathological data were collected and merged with patient questionnaires. No cases reported a positive family history of early onset CRC or multiple familial cases that might indicate an inherited dominant CRC gene. A modified version of Duke's staging system was used. Surgical records identified 8 subsites: cecum, ascending colon, transverse colon, descending colon, sigmoid colon, rectosigmoid junction, rectum and anal canal. Proximal tumors were defined as including the right side of the colon and descending colon up to the sigmoid colon.

The control group consisted of consecutively recruited patients presenting to the Trauma Service of the Hospital Clinic during the same time period as case accrual. This service deals with approximately 2,000 admissions /year of patients living in the same metropolitan area (Barcelona) as the cases studied.To facilitate sampling, patients whose pre-operatory study (including blood analysis) was performed in the outcare service were excluded. Only patients between ≥40 years of age and <90 years were invited to participate in the study, following the same procedure as for cases. The same epidemiological data and blood samples were collected from controls as for cases. Most individuals of the control group were admitted for articular hip or knee joint replacement, pelvis fracture or injury to upper or lower ribs. Controls were excluded if they reported a history of malignancy.

Dietary patterns. These were analyzed by a semiquantitative food frequency questionnaire (FFQ) (15) designed to address epidemiological and nutritional hypotheses within the population of different regions of Spain, specifically conceived to be used in a population-based case-control study on breast and colorectal cancer. This FFQ includes 118 food items structured into 11 categories (dairy foods, eggs meat and fish, vegetables, fruits, legumes, cereals, oils and fats, sweets and baked foods, beverages, precooked or prepacked foods and miscellaneous). Cases and controls were contacted by trained research assistants who requested an appointment for an in-person interview. The interviews were followed by an informal discussion with the participants to verify the performance of the questionnaire in terms of clarity and comprehension. The research assistants were instructed to make an operational estimate of the described portion sizes. The nutrient composition of the 24-hour recalls was determined using computerized dietary analysis software (16) adapted for our study with standard Spanish food composition tables (16-19).

Ki-ras mutation detection. DNA was isolated from tumor specimens using standard methods employing RNAase, proteinase K, chloroform/isoamyl alcohol extraction and ethanol precipitation. DNA was quantified by Hoescht 33258 fluorimetry (Hoefer Scientific, San Francisco, CA, USA). All analyses were carried out on coded samples and the analyst was blind to the case-control status. Ki-*ras* mutations in codon 12 and 13 were detected by a nested PCR method in which the second round PCR used mismatched primers that introduced restriction sites into products derived from wild-type alleles. Mutations identified by this screening method were then sequenced using dot-blot hybridization or by direct sequencing. First round primers and conditions were taken from Nelson (20), sense 5'-CATGTTCTAA TAAGTCACA-3', antisense; 5'-TCAAAGAATGGTCCTGCACC-3' (GeneBank accession no. L00045). PCR reactions (25 ml volume) were carried out with 50 ng of tumor DNA and the following cycling conditions (15 cycles) with a Perkin Elmer 9600 thermocycler: preheating at 94°C for 2 min, denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 10 min. The second round PCR was described by Jacobson and Mills (21). Two μl of the first round PCR product was added to the second round PCR (100 μl volume) and amplified with a screening primer (sense) for either codon 12: (5'-ACTGAATATAAACTTGTGGTAGTTGGACCT-3'), or codon 13 (:5'-CTGAATATAAACTTGTGGTAGTTCCAGCTGGT-3'). The same antisense primer as used in the first round PCR was used for both codon 12 and 13 screening. PCR was repeated for 35 cycles under the following conditions: 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, final extension at 72°C for 10 min. After PCR, 16 μl of the second round PCR product was digested with either BstN1 (codon 12) or Pflm1 (codon 13) for 4 h. Digests were analyzed using 3.5% agarose gels. PCR products resistant to enzyme digestion indicated the presence of base pair substitution. For each set of samples a positive control DNA (SW480) containing a codon 12 mutation was also run to provide quality control on the amplification and restriction enzyme digestion. After tumors were identified as having mutations in codon 12 or 13 they were then analyzed using the human ras MUTALYZER probe panel and dot-blot hybridization according to the manufacturer's instructions (Clontech, Palo Alto, CA, USA). If the products were very strong, they were sequenced directly. For direct sequencing, the second round products were gel purified (Qiagen, Valencia, CA, USA) and sequenced with the antisense primer using an ABI 373 automated sequencer. To confirm the accuracy of the dot blot results, a total of 31 samples were directly sequenced and dot blotted.

Statistical analyses. Data were analyzed using SPSS 6.1.2 (statistical analysis software). Means and standard deviations were computed for continuous variables. Micronutrients were categorized by tertiles and studied for their association with CRC, through multivariate analysis,

Table I. *Demographic and clinical-pathological characteristics of controls and CRC patients.*

Variable	Controls	All CRC	Molecular CRC	Total Ki-*ras* mutation[a]	K12 mutation[b]	K13 mutation[c]
n	296	247	117	49/117 (41.8%)	35/117(29.9%)	14/117(11.9%)
Mean age	61.9±11.6	70.4±11.2	71.0±11.1			
Men	111/296(37.5%)					
	131/247(53.0%)	61/117(52.1%)	27/61(44.2%)	21/61(34.4%)	6/61(9.8%)	
Women	185/296(62.5%)					
	116/247(46.9%)	56/117(47.8%)	22/56(39.2%)	14/56(25%)	8/56(14.3%)	
Stage		#	##			
A		4/236(1.6%)	2/116(1.7%)			
B		96/236(40.2%)	46/116(39.6%)	23/48(47.9%)	18/48(37.5%)	5/48(10.4%)
C		67/236(28.3%)	33/116(28.4%)			
D		69/236 (29.6%)	35/116(30.1%)	26/68(38.2%)	17/68(25%)	9/68(13.2%)
Differentiation		*	**			
Mod to well		191/208(91.8%)	95/107(88.7%)	42/95(44.2%)	31/95(32.6%)	11/95(11.5%)
Poor		17/208(8.1%)	12/107(11.2%)	4/12(33.3%)	2/12(16.6%)	2/12(16.6%)
Location						
Cecum		20/247(8.0%)	14/117(11.9%)			
Ascending		34/247(13.7%)	12/117(10.2%)			
Transverse		19/247(7.6%)	9/117(7.6%)			
Descending		9/247(3.6%)	5/117(4.2%)			
Total Proximal		82/247(33.1%)	40/117(34.1%)	17/40(42.5%)	13/40(32.5%)	4/40(10%)
Sigmoid		87/247(35.2%)	40/117(34.1%)			
Sigmorectal		19/247(7.6%)	12/117(10.2%)			
Rectal		59/247(23.8%)	25/117(21.3%)			
Total Distal		165/247(66.8%)	77/117(65.8%)	32/77(41.5%)	22/77(28.5%)	10/77(12.9%)

[a] number of patients in the molecular CRC group with Ki-*ras* mutations, [b] in codon 12, [c] in codon 13.There are some cases for which we have no clinical information (missing: # 11 cases, ## 1 case; *34 missing, **10 missing).

each of them being adjusted for age and K-calories. Confidence limits for the adjusted odds ratio were calculated with the associated logistic coefficients and standard errors. The micronutrients which were significantly associated with protection against or risk of CRC were studied by comparing means (Student's *t*-test and *U*-Mann Whitney).

Results

The demographic characteristics of controls and clinical-pathological data of CRC patients are shown in Table I. The distribution of the sexes with more male cases than females in both case groups was identical to the 53% males among incident CRCs reported by the population-based Cancer Registry of Catalonia (Tarragona, Spain). The control group, however, contained relatively more women. This is most probably due to the hospital-based recruitment of patients seeking medical treatment for hip, pelvic and other fractures, which are more prevalent among postmenopausal women compared with older men. The control group was about 10 years younger than the CRC group. The overall and molecular CRC groups were very similar in terms of the distributions of Duke's stage A-D, percentage of poor histopathological differentiation and anatomic location of the primary tumor. Approximately 57.6% of the patients had Duke's stage C or D disease and 8.1% presented with tumors classified as poorly-differentiated. In addition, men ≤70 years old more frequently had distal tumors (all CRC: 79.4%; molecular CRC: 93.1%) and women aged > 70 years presented with proximal tumors in 46.5% and 62.5% of cases within the overall CRC group and molecular CRC group, respectively.

Ki-*ras* mutations were observed in 41.8% of all cases; these were located in codon 12 in 29.9% of patients and in codon 13 in 11.9% of patients (Table I). Although the prevalence of Ki-*ras* mutations was higher in men than in women (34.4% *versus* 25% mutant at codon 12), the difference was not statistically significant. Poorly-differentiated tumors had roughly half the number of codon 12 mutations compared with moderate- or well-differentiated tumors but once again the difference was not statistically significant.

Table II shows the average daily intake of nutrients for colorectal patients and controls. Differences between means showed a lower consumption of fiber, calcium, potassium, iron, vitamins B1, B2, A, D, E, folates and alcohol in the CRC groups (overall and molecular) compared to controls.

Table III shows the results of the multivariate analysis for selected micronutrients, in two models. The first model compares controls *vs* cases, while the second compares controls *vs* the molecular CRC subgroup. Similar results were

Table II. *Average daily intake of nutrients for controls, colorectal cancer patients (CRC) and for the molecular subgroup of CRC patients, estimated from a food frequency questionnaire.*

		Controls n=296 ALL MEN WOMEN	CRC n=246 ALL MEN WOMEN	p^1	Molecular CRC n=117 ALL MEN WOMEN	p^2
Kcalories	Kcal	2286.51±569.6 2443.15±630.7*** 2192.53±508.6	2283.14±565.4 2373.00±605.3 2180.70±499.3		2257.20±598.4 2332.10±624.9 2173.96±561.6	
Fats	g	94.05±25.5 97.51±26.9 91.98±24.6	93.45±28.3 95.60±30.3 91.00±25.7		93.26±30.2 94.06±33.6 92.37±26.2	
Polyunsaturated	g	11.23±5.0 11.87±4.9 10.84±5.1	10.88±5.0 11.57±5.4 10.09±4.3		10.35±4.5 10.80±5.1 9.85±3.8	
Monounsaturated	g	40.85±10.2 42.35±11.0 39.95±9.7	41.03±11.2 42.32±12.1 39.56±10.0		41.31±11.7 42.02±13.2 40.51±10.0	
Saturated	g	28.11±11.6 28.17±12.5 28.07±11.1	28.72±13.4 29.05±14.2 28.34±12.4		29.06±14.9 29.05±16.3 29.07±13.4	
Cholesterol	mg	456.99±185.4 486.37±208.0* 439.35±168.6	448.36±203.5 481.40±244.0 410.52±135.6		468.42±251.0 513.97±316.3 417.91±134.2	
Protein	g	81.45±20.3 80.33±23.0 82.12±18.5	79.68±19.8 81.07±20.9 78.10±18.5		78.75±20.8 79.71±21.9 77.67±19.7	
Vegetable	g	25.64±7.9 25.84±7.9 25.52±7.9	25.71±7.8 26.29±7.4 25.04±8.3		25.37±7.6 25.78±7.1 24.92±8.1	
Non Vegetable	g	50.68±16.2 49.32±18.4 51.49±14.7	48.83±15.6 49.51±17.1 48.05±13.7	0.06	48.29±16.4 48.76±17.9 47.77±14.8	
Carbohydrates	g	256.14±75.5 265.89±75.7 250.29±75.0	260.60±80.9 268.21±88.3 251.94±71.0		253.40±77.7 259.83±76.6 246.27±78.9	
Fiber	g	18.11±6.3 17.32±5.5 18.59±6.8	16.47±6.2 16.74±6.8 16.15±5.5	0.003 0.001	15.80±5.2 15.58±4.9 16.04±5.6	0.001 0.04 0.01
Calcium	mg	865.29±416.4 778.81±487.6** 917.17±358.6	823.09±323.2 823.60±359.8 822.52±277.3	 0.01	784.90±311.5 770.95±347.8 800.56±267.9	0.06 0.02
Phosphorus	mg	1417.72±442.0 1384.23±496.2 1437.81±406.2	1386.99±415.0 1387.70±428.8 1386.11±400.4		1349.79±413.2 1331.15±415.5 1370.46±413.5	
Potassium	mg	3173.47±1568.9 2993.00±1423.3 3281.46±1644.3	3044.97±2136.3 3152.80±2652.1 2922.08±1326.0	 0.04	2778.74±1065.4 2728.14±1049.7 2834.44±1089.5	0.01 0.05 0.05

Table II. *Continued.*

		Controls n=296 ALL MEN WOMEN	CRC n=246 ALL MEN WOMEN	p^1	Molecular CRC n=117 ALL MEN WOMEN	p^2
Sodium	mg	2010.49±649.2 2162.54±674.5** 1919.27±617.7	1938.36±641.5 2013.36±686.9 1852.92±576.5		1928.56±692.0 2002.45±750.6 1846.61±617.0	
Iron	mg	12.38±3.1 12.42±3.3 12.35±2.9	11.96±3.3 12.31±3.5 11.55±2.9	 0.02	11.76±3.0 11.92±3.1 11.59±3.0	
Magnesium	mg	354.96±137.2 330.68±137.7** 369.53±135.1	340.19±124.8 337.04±121.3 343.76±129.2		334.18±118.8 326.57±115.4 342.61±123.0	
Zinc	mg	9.07±3.5 9.24±4.4 8.96±2.9	9.09±4.8 9.50±5.9 8.54±2.9		9.35±6.4 9.89±8.2 8.75±3.4	
Vitamin B1	mg	1.25±0.3 1.26±0.3 1.25±0.3	1.20±0.3 1.24±0.3 1.16±0.2	0.06 0.01	1.16±0.3 1.16±0.3 1.15±0.3	0.007 0.06 0.05
Vitamin B2	mg	1.98±0.7 1.93±0.8 2.01±0.6	1.91±0.6 1.97±0.7 1.84±0.4	 0.01	1.88±0.6 1.91±0.7 1.85±0.5	
Vitamin B6	mg	2.01±0.4 1.99±0.4 2.02±0.4	1.98±0.5 2.01±0.5 1.94±0.5		1.97±0.6 1.97±0.6 1.96±0.5	
Vitamin B12	mg	7.95±4.0 8.70±4.9* 7.49±3.3	7.67±4.7 8.22±5.8 7.04±2.9		8.00±6.0 8.61±7.8 7.33±3.0	
Folates	mg	306.44±87.1 300.40±79.1 310.01±91.6	288.10±89.1 293.82±87.9 281.60±90.4	0.01 0.009	281.20±83.9 282.69±77.7 279.55±91.0	0.008 0.03
Vitamin C	mg	101.87±43.7 94.04±42.1** 106.57±44.0	96.87±44.8 95.31±46.5 98.65±42.9		94.45±42.1 91.06±40.2 98.20±44.3	
Vitamin A	µg	3505.15±1647.2 3413.72±1642.3 3560.00±1652.1	3050.55±1636.2 3102.27±1681.5 2991.63±1588.4	0.001 0.004	1801.05±1801.7 3008.91±1852.1 3118.88±1759.2	0.02
Vitamin D	µg	4.03±2.5 4.10±2.2 3.99±2.7	3.55±2.5 3.45±2.0 3.65±3.0	0.02 0.02 	3.51±2.3 3.52±2.1 3.51±2.5	0.05
Vitamin E	mg	9.19±3.18 9.22±3.2 9.17±3.1	8.65±3.2 8.78±3.3 8.51±3.2	0.05 	8.21±3.0 8.16±3.1 8.26±2.9	0.005 0.04 0.05
Alcohol	g	11.29±22.6 23.98±31.8*** 3.67±7.9	9.94±18.4 14.91±22.7 4.28±8.9	 0.01 	10.31±19.4 15.40±23.7 4.56±10.7	

p^1 Significant differences (Student's *t*-test or *U*-Mann Whitney) for controls *vs* cases and p^2, significant differences for controls *vs* molecular CRC. Significant differences between men and women; (Student's *t*-test or *U*-Mann Whitney) $*p<0.05$; $**p<0.01$; $***p<0.001$.

Table III. *Summarized results for multivariate analysis of the micronutrients-dependent risk for CRC.*

	Control *vs* CRCs[2] OR (CI)			Control *vs* Molecular CRCs[3] OR (CI)		
Level intake[1]	High	Medium	Low	High	Medium	Low
Vitamin E Low Intake≤7.06 mg/day Medium Intake>7.06 mg/day≤10 mg/day High Intake>10 mg/day	1	0.7(0.4-1.2)	1.6(0.9-2.8)	1	0.8(0.4-1.4)	2.3(1.2-4.6)*
Vitamin D Low Intake≤1.90 μg/day Medium Intake>1.90 μg/day ≤5.65 μg/day High Intake > 5.65 μg/day	1	1.5(0.9-2.5)	1.9(1.1-3.3)*	1	1.5(0.8-2.8)	2(1.1-4.2)*
Vitamin B1 Low Intake ≤ 1.03 mg/day Medium Intake>1.03 mg/day≤1.42 mg/day High Intake > 1.42 mg/day	1	0.9(0.5-1.6)	1.7(0.9-3.1)	1	1(0.5-2.1)	2.5(1.2-5.1)*
Vitamin A Low Intake≤ 2296 μg/day Medium Intake>2296 μg/day≤4320 μg/day High Intake > 4320 μg/day	1	1.3(0.8-2.2)	2.4(1.3-4.2)**	1	1.2(0.6-2.4)	2.5(1.2-5.1)*
Folates Low Intake≤ 252 μg/day Medium Intake> 252 μg/day ≤ 346 μg/day High Intake > 346 μg/day	1	1.1(0.6-1.6)	1.8(1.1-3.1)*	1	0.8(0.5-1.6)	2(1.1-3.9)*
Fiber Low Intake≤ 14 g/day Medium Intake> 14 g/day ≤ 20 g/day High Intake > 20 g/day	1	1.5(0.9-2.4)	2.4(1.1-4.1)**	1	1.3(0.7-2.4)	2.7(1.4-5.1)**
Calcium Low Intake≤ 610 mg/day Medium Intake>610 mg/day≤1044 mg/day High Intake > 1044 mg/day	1	1.2 (0.7-1.9)	1.8(1.1-3.2)*	1	1.3(0.7-2.5)	2.3(1.1-4.6)*
Potassium Low Intake≤ 2187 mg/day Medium Intake>2187mg/day≤3690mg/day High Intake > 3690 mg/day	1	1.1(0.7-1.7)	1.4(0.8-2.5)	1	1.3(0.7-2.3)	1.8(0.9-3.6)
Iron Low Intake≤ 10 mg/ day Medium Intake> 10 mg/day ≤ 14 mg/day High Intake > 14 mg/day	1	1.1(0.6-1.7)	1.8(0.9-3.2)	1	1(0.5-1.8)	2.1(0.9-4.5)
Vitamin B2 Low Intake≤ 1.49 mg/day Medium Intake>1.49 mg/day≤2.27 mg/day High Intake > 2.27 mg/day	1	0.9(0.5-1.5)	1.6(0.9-2.9)	1	0.7(0.4-1.4)	1.8(0.9-3.8)

** $p \leq 0.01$ * $p < 0.05$

[1] categorized by tertiles. Cut-off point for each micronutrient is described below in the table

[2] multivariate analysis adjusted by age and energy contribution of each nutrient to the risk of CRC (using CRC group).

[3] multivariate analysis adjusted by age and energy contribution of each nutrient to the risk of CRC using the molecular CRC group.

Table IV. *Selected micronutrient intake in molecular Ki-ras subgroups of CRC.*

Micronutrient	K12(+) n=35	K12(-) n=81	p^* a	b
Fiber (g/d)	15.2 ± 4	16.0 ± 5	0.01	0.008
Folate (μg/d)	271.8 ± 78	285.2 ± 86	0.02	0.05
Vitamin A (μg/d)	2847.9 ± 1633	3153 ± 1871	0.02	ns
Vitamin B1 (mg/d)	1.11 ± 0.29	1.18 ± 0.31	0.01	ns
Vitamin D (μg/d)	3.03 ± 2	3.73 ± 2	0.02	ns
Vitamin E (mg/d)	8.14 ± 2.7	8.24 ± 3.1	0.06	0.01
Potassium (mg/d)	2713.17 ± 953.7	2806.79 ± 1114.7	0.06	0.04
Calcium (mg/d)	759.53 ± 343.5	795.99 ± 298.2	ns	ns
Iron (g/d)	11.19 ± 2.7	12.01 ± 3.1	0.03	ns
Micronutrient	K13(+) n=14	K13(-) n=102	p^* c	d
Fiber (g/d)	18.7 ± 5	15.4 ± 5	ns	0.000
Folate (μg/d)	323.1 ± 94	275.4 ± 81	ns	0.002
Vitamin A (μg/d)	3975 ± 2169	2935 ± 1720	ns	0.003
Vitamin B1 (mg/d)	1.32 ± 0.26	1.14 ± 0.31	ns	0.002
Vitamin D (μg/d)	3.27 ± 1	3.55 ± 2	ns	ns
Vitamin E (mg/d)	8.04 ± 2.1	8.23 ± 3.1	ns	0.008
Potassium (mg/d)	2647.01 ± 875.5	2796.59 ± 1091.3	ns	0.02
Calcium (mg/d)	884.74 ± 351.0	771.30 ± 305.1	ns	0.03
Iron (g/d)	12.21 ± 2.7	11.70 ± 3.1	ns	0.05

*Student's *t*-test or *U*-Mann Whitney analysis comparing controls with either (a) molecular CRC with Ki-*ras* mutation in codon 12, or (b) molecular CRC without Ki-*ras* mutation in codon 12; (c) molecular CRC with Ki-*ras* mutation in codon 13, or (d) molecular CRC without Ki-*ras* mutation in codon 13.

found in both models, showing that no bias was introduced in the selection of this subgroup. Fiber appeared to be most protective on comparing controls either *vs* all cases or *vs* the molecular subgroup of cases. Greater vitamin E, D, B1, A, folate and calcium intakes were also associated to a greater or lesser extent with reduced risk of CRC. Potassium, iron and vitamin B2 showed a non significant trend towards a reduction of the CRC risk. Alcohol was not correlated with reduced or increased risk for CRC.

Table IV shows the mean intake of these micronutrients in each molecular subgroup as determined by the presence or not of Ki-*ras* mutations. Patients with mutations in codon 12 (K12) consumed significantly less vitamin A (p=0.02), B1 (p=0.01), D (p=0.02) and iron (p=0.03) than controls, whereas patients without these mutations had similar intakes of these specific micronutrients as controls (Figure 1). No differences between the intake of fiber, folates, vitamin E or potassium were found among patients with and without a mutation in codon 12. Mutation in codon 13 (K13) was not significantly associated with any micronutrient lack. Deficits in vitamin E, D, B1 and A, folates , fiber and calcium were observed in the group of patients without codon 13 mutations. Lack of calcium consumption was not associated with any molecular alteration. Multivariate analysis for each nutrient, adjusted by age and energy, and comparing controls with each molecular subtype of CRC, did not give a significant OR.

Discussion

The present research used epidemiological data from a molecular CRC study which included different risk polymorphisms (13, 14) and the molecular characteristics of tumors by studying Ki-*ras* mutations. Therefore, the research design is not equivalent to that of a study of diet and cancer risk but rather is geared toward collecting data with the secondary aim of examining possible relationships between micronutrients and molecular parameters.

The results regarding the consumption of different nutrients in our country's population shown in Table II are similar to those published by other Spanish groups (22). The clinical-pathological characteristics in our CRC group and the prevalence of tumor Ki-*ras* mutations are similar to those reported in Spain (10) and for other series (23, 24).

Prior to studying the possible associations between nutrients and molecular variables, it was necessary to perform a multivariate analysis adjusted for age and energy, as these two components have traditionally been used in this type of analysis; however in our study we found no significant differences between cases and controls with these two parameters. The nutrients identified as being at "risk" in this statistical analysis were those which were subsequently studied with respect to Ki-*ras* mutations. Our finding that a lower intake of vitamins E, D, B1, A, folate, fiber and calcium is associated with increased risk for CRC has also been described by other authors.

Dietaty fiber is widely considered to protect against colorectal cancer (25). Since the pioneering studies of Burkitt (26) it has generally been believed that a high intake of dietary fiber is beneficial, although there have recently been debates regarding which type of fiber is most beneficial. It is also well known that folate deficiency or simply low levels of folate intake may be related to the risk of colorectal cancer (27). Several epidemiological studies have reported calcium as having a protective effect against colorectal cancer (28). In a recently published analysis of cases from the Nurses' Health Study and the Health Professionals Follow-up Study, calcium intake reduced the risk of distal but not proximal colon cancer (29). With respect to vitamin D, dietary intake may reduce the risk of large bowel cancer, particulary rectal cancer (30). An inverse geographical relationship between latitude and colon cancer incidence has also been reported: increased exposure to sunlight reduced the risk of colorectal cancer, presumably through mechanisms involving vitamin D utilization and calcium (31).

In general the consumption of multivitamins has been associated with greater protection against colorectal cancer

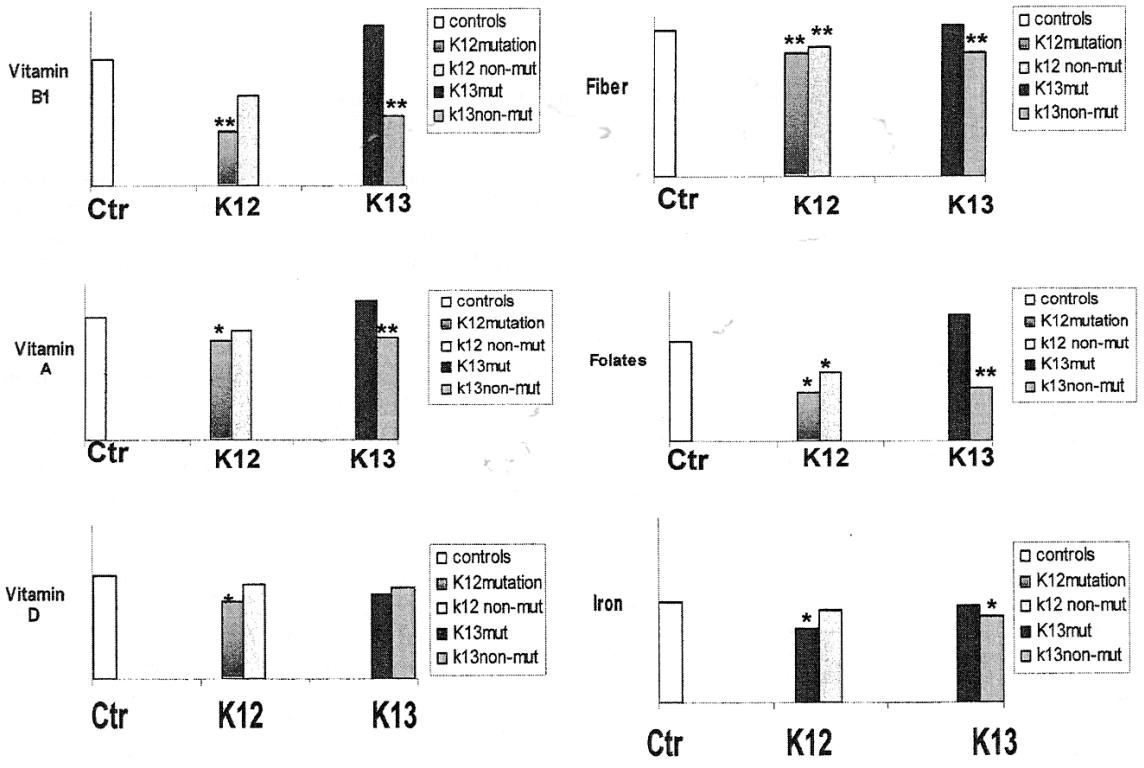

Figure 1. *Average of selected nutrient intake in molecular Ki-ras subgroups and comparison with controls* * $p \leq 0.05$ *Comparison with controls intake.* ** $p \leq 0.01$ *Comparison with controls intake*

(32). An inverse association between the intake of retinol, thiamine (vitamin B1) or antioxidant micronutrients (vitamin E) and the occurrence of colorectal cancer has also been reported (33). Vitamin E is the major radical trap in lipid membranes and has been used clinically in a variety of oxidation-related diseases. Protection against radiation-induced DNA damage and mutation and dimethylhydrazine-induced carcinogenesis have also been observed (34). However findings from studies on vitamin E may show conflicting results (35-38). Bostick *et al.* found an inverse association between vitamin E and cancer that was stronger in younger age groups (35). Vitamin A and retinoids in general are physiological regulators of cell growth and differentiation and they are used in the treatment or chemoprevention of several malignant diseases (39, 40); therefore it is not surprising to find this vitamin among the protection factors for CRC.Vitamin B1 (thiamine) had the opposite (and unclarified) effect in experimental models of cell proliferation. Although it is metabolized to thiamine pyrophosphate, the cofactor of transketolase (which is involved in ribose synthesis and necessary for cell replication) (41), high doses of thiamine had the opposite effect and caused almost 35% inhibition of tumor growth.

Studies of iron consumption and CRC risk suggest it to be more of a risk factor than a protective one; this is because haem-iron can increase the carcinogenic fecal N-nitrous compounds in the bowel. However, excess iron is associated with a greater risk of proximal tumors (42) which constitute the minority of our group (38%).

One of the first genes to be linked to the pathogenesis of colorectal carcinomas in humans was the Ki-*ras* gene (43). Mutations in this gene occur in both adenomas and carcinomas and are found in approximately 40% of advanced colorectal neoplasms. The frequency and spectrum of Ki- *ras* mutations in human cancers vary greatly and may be caused by exposure to different genotoxic agents. Codon 12 is the predominate target for mutations in Ki-*ras* in CRC, here accounting for 70% of the observed alterations. This is similar to lung cancer where codon 12 mutations comprise over 95% of Ki-*ras*

mutations, being closely linked with cigarette exposure, asbestos and female gender. The relatively lower number of codon 13 mutations in human cancers is not understood; however, the chemical reactivity of mutagens with specific nucleotides within *ras*, the repair efficiency toward different DNA modifications and the resulting mutational spectra have been shown to be highly sequence-dependent (44-47). This is why we studied micronutrient consumption for patients with mutations in one or the other codon separately. The fact that mutations in codon 13 have not been associated with any specific deficit may be due to the small number of tumors presenting this mutation. The Ki-*ras* mutations in codon 12 are particularly interesting, since in the group with absence of mutation the reduction in the consumption of specific nutrients was not significantly different from controls. Thus, these mutations determine most of the differences in vitamin A, B1, D and iron intakes between control and cases. The precise mechanism by which these nutrients may protect against Ki-*ras* mutations is uncertain. It may be that the antioxidant effect of some of these vitamins hinders the access of mutagenic substances which are reactive with this oncogene. However, it is also possible that these deficiencies impair DNA repair mechanisms in the colon. Therefore cells can develop genomic instability and rapidly accumulate somatic mutations or loss of short segments of alleles within the oncogene. Indeed both retinoids and vitamin D are necessary to maintain correct cell growth and differentiation (39,40, 48). Receptors for vitamin D have been reported on human colorectal neoplasms, suggesting that it may play a role in colorectal cancer development (48); however, as yet no direct relationship with the Ki-*ras* oncogene has been identified.

Some of the previously reported associations between nutrients and Ki-*ras* mutations were not found in the present study. For example, whereas other authors have reported a protective effect for calcium against Ki-*ras* mutations (10), in our study calcium consumption was not related to the presence of such mutations.

Epidemiological and experimental studies point to dietary components as important factors in the etiology of sporadic colorectal carcinoma. Differences in diet may explain, in part, the 20-fold geographical variation in incidence rate observed for this neoplasm. Little is known about the effect of nutrients upon the specific genetic alterations found in tumors. Our findings support results of published data on the potential protective role of specific nutrients in colorectal carcinogenesis. If the observed associations are correct, it would seem that the protective effect of these nutrients in CRC may be mediated through their effect on the Ki- *ras* mutation rate. However, given the limited information currently available on associations between specific genetic mutations in colon tumors and diet, these findings should also be viewed as hypothesis generating. Future studies, particularly population studies with large sample sizes, should be able to confirm this association and more adequately explore the role of other host and environmental risk factors in terms of how they relate to Ki-*ras* mutation rates.

The significant association between diet and the risk of a specific subtype of CRC (codon 12 mutant) highlights once more the need to incorporate more sophisticated methods for sub-classifying CRC into etiologically more homogeneous groups, perhaps more closely linked to the environmental causes of the disease. Such an approach, when integrated with analytical epidemiological designs, may reveal distinct etiological pathways and risk factors for CRC, which is increasing in many parts of the world (including Spain) that have historically experienced low and moderate rates of the disease.

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