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Colorectal cancer risk and nitrate exposure through drinking water and diet

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Ingested nitrate leads to the endogenous synthesis of N-nitroso compounds (NOCs), animal carcinogens with limited human evidence. We aimed to evaluate the risk of colorectal cancer (CRC) associated with nitrate exposure in drinking water and diet. A case-control study in Spain and Italy during 2008-2013 was conducted. Hospital-based incident cases and population-based (Spain) or hospital-based (Italy) controls were interviewed on residential history, water consumption since age 18, and dietary information. Long-term waterborne ingested nitrate was derived from routine monitoring records, linked to subjects' residential histories and water consumption habits. Dietary nitrate intake was estimated from food frequency questionnaires and published food composition databases. Odd ratios (OR) were calculated using mixed models with area as random effect, adjusted for CRC risk factors and other covariables. Generalized additive models (GAMs) were used to analyze exposure-response relationships. Interaction with endogenous nitrosation factors and other covariables was also evaluated. In total

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1,869 cases and 3,530 controls were analyzed. Average waterborne ingested nitrate ranged from 3.4 to 19.7 mg/day, among areas. OR (95% CIs) of CRC was 1.49 (1.24, 1.78) for >10 versus ≤5 mg/day, overall. Associations were larger among men versus women, and among subjects with high red meat intake. GAMs showed increasing exposure-response relationship among men. Animal-derived dietary nitrate was associated with rectal, but not with colon cancer risk. In conclusion, a positive association between CRC risk and waterborne ingested nitrate is suggested, mainly among subgroups with other risk factors. Heterogeneous effects of nitrate from different sources (water, animal and vegetables) warrant further research.

What's new?

Nitrate ingested in food and water can react with amines and amides in the gastrointestinal tract, leading to the formation of N-nitroso compounds (NOCs), which are carcinogenic in animals. In humans, nitrate and several NOCs are probable carcinogens. The aim of the present investigation, a case–control study in Europe, was to examine links between nitrate intake and colorectal cancer (CRC). The findings indicate that CRC risk is increased for waterborne nitrate intake at levels below current international guidelines, particularly in subgroups with other risk factors. Nitrate intake from animal sources was further associated with increased rectal cancer risk.

Introduction

Nitrate is a widespread contaminant in drinking water due to the overuse of fertilizers in agriculture[1] and urban sewage.[2] Expensive and infrequently used methods such as reverse osmosis are necessary to effectively remove nitrate from drinking water.[3] In addition, nitrate is a main dietary component of vegetables, and an approved food additive for preserved meat, together with nitrite.[4]

Nitrate ingestion through diet and drinking water are the main routes of human exposure. Ingested nitrate is reduced to nitrite, which subsequently reacts with amines and amides to produce N-nitroso compounds (NOCs) in the gastrointestinal system. The intake of vitamins C and E may inhibit endogenous nitrosation, whereas meat intake and chronic gastrointestinal acidic or inflammatory conditions, may increase it.[5] Additionally, exogenous NOCs are ingested through processed meat, canned or cured food, alcohol and tobacco smoking.[6] NOCs are carcinogenic in several animal species,[7] but human evidence is limited, therefore nitrate is classified as probable human carcinogen (group 2A) under conditions resulting in endogenous nitrosation.[8]

Colorectal cancer (CRC) is one of the most frequent cancers worldwide, representing 10% of the global cancer incidence. More than 1 million new cases and 694,000 deaths are registered annually in both sexes.[9] The high intake of energy, red or processed meat[10] and alcohol, as well as physical inactivity and obesity, are established risk factors.[11] Increased CRC risk has been suggested with dietary nitrite[12] or dietary NOCs.[13,14] Recently, a prospective study found an increased risk among subjects with high dietary nitrate and low vitamin C intake.[15] However, few studies have evaluated the risk of CRC associated with nitrate in drinking water. Existing evidence provided by case–control or cohort studies is inconsistent,[5,16] particularly for levels below the current regulatory limit (50 mg/L of nitrate as NO_3^- in the European Union, or 10 mg/L as NO_3-N in the United States),[17] which is a common scenario in high-income countries.

We evaluated the association between CRC risk and the exposure to nitrate through drinking water and diet, taking into account endogenous nitrosation factors and other covariates.

Methods

Study design and population

We pooled data from two case-control studies conducted in Spain (the Spanish Multi-case Control study on Cancer, MCC-Spain)[18] and Italy (part of the European Union Project on Health Impacts of long-term exposure to Disinfection by-products in Drinking Water, HI-WATE),[19] between 2008 and 2013. Study areas comprised eleven provinces (nine from Spain, two from Italy) (see Table 1). CRC cases were identified as soon as possible after the diagnosis through active searches including periodical visits to the hospital departments (*i.e.*, oncology, gastroenterology, general surgery, radiotherapy and pathology). Participant hospitals (17 in Spain, 10 in Italy) were the reference centers for oncologic diseases in each study area. Only CRC cases diagnosed within the recruitment period, with histological confirmation (ICD-10 codes: C18, C19, C20, D01.0, D01.2), without previous cancer history, aged 20-85 years, living in the hospitals' catchment areas, and being able to answer an epidemiological questionnaire, were enrolled. Controls were hospital-based (Italy) or population-based (Spain), and were frequency matched to cases by sex, age and residence area. Hospital-based controls were randomly selected among patients admitted to the same hospitals as cases for acute, non-chronic diseases, unrelated to alcohol, tobacco, dietary habits or to known CRC risk factors (52.2% had acute surgical conditions, 9.0% non-traumatic orthopedic disorders, 6.0% trauma and 32.8% other illnesses). Population-based controls were randomly selected

Table 1. Characteristics of the study population included in the analyses of waterborne ingested nitrate: 1,869 cases of colorectal cancer (1,285 colon and 557 rectum)[1] and 3,530 controls

	Cancer cases				
	Colon *n* (%)	Rectum *n* (%)	Colorectal *n* (%)	Controls *n* (%)	*p* values[2]
Study area					
Spain					
Asturias	38 (3.0)	21 (3.8)	59 (3.2)	202 (5.7)	
Barcelona	430 (33.4)	189 (33.9)	629 (33.6)	910 (25.8)	
Cantabria	79 (6.2)	40 (7.2)	119 (6.4)	279 (7.9)	
Gipuzkoa	83 (6.5)	24 (4.3)	107 (5.7)	333 (9.4)	
León	162 (12.6)	77 (13.8)	243 (13.0)	352 (10.0)	
Madrid	143 (11.1)	62 (11.1)	206 (11.0)	658 (18.6)	
Murcia	13 (1.0)	12 (2.2)	25 (1.3)	39 (1.1)	
Navarra	72 (5.6)	24 (4.3)	98 (5.2)	230 (6.5)	
Valencia	52 (4.0)	24 (4.3)	76 (4.1)	131 (3.7)	
Italy					
Milan	118 (9.2)	56 (10.0)	184 (9.8)	270 (7.6)	
Pordenone/Udine	95 (7.4)	28 (5.0)	123 (6.6)	126 (3.6)	<0.001
Sex					
Men	784 (61.0)	382 (68.6)	1,184 (63.4)	1,840 (52.1)	
Female	501 (39.0)	175 (31.4)	685 (36.6)	1,690 (47.9)	<0.001
Age (quartiles)					
≤57 years	226 (17.6)	109 (19.6)	341 (18.2)	947 (26.8)	
58-65 years	294 (22.9)	151 (27.1)	449 (24.0)	870 (24.6)	
66-72 years	335 (26.1)	110 (19.8)	455 (24.3)	833 (23.6)	
>72 years	430 (33.5)	187 (33.6)	624 (33.4)	880 (24.9)	<0.001
Education					
<Primary school	334 (26.0)	147 (26.4)	484 (25.9)	585 (16.6)	
Primary school	478 (37.2)	222 (39.9)	707 (37.8)	1,176 (33.3)	
Secondary school	345 (26.8)	147 (26.4)	505 (27.0)	1,101 (31.2)	
University	128 (10.0)	41 (7.4)	173 (9.3)	668 (18.9)	<0.001
Physical activity (METs hour/week)[3]					
<8 (<8.5)	862 (67.1)	376 (67.5)	1,252 (67.0)	2,266 (64.2)	
8–16 (8.5–34.4)	178 (13.8)	80 (14.4)	266 (14.2)	550 (15.6)	
>16 (>34.4)	245 (19.1)	101 (18.1)	351 (18.8)	714 (20.2)	0.121
Smoking[4]					
Never	549 (43.2)	202 (36.3)	761 (41.0)	1,535 (43.6)	
Ever	723 (56.8)	354 (63.7)	1,093 (59.0)	1,986 (56.4)	0.073
Non-steroidal anti-inflammatory drugs use[4]					
Never	848 (68.4)	371 (68.2)	1,241 (68.6)	2,134 (62.6)	
Ever	391 (31.6)	173 (31.8)	569 (31.4)	1,274 (37.4)	<0.001
Oral contraceptives use[4,5]					
Never	355 (71.1)	124 (72.5)	486 (71.6)	940 (55.9)	
Ever	144 (28.9)	47 (27.5)	193 (28.4)	742 (44.1)	<0.001
Colorectal cancer in first degree relative[4]					
No	1,005 (83.8)	450 (85.6)	1,479 (84.5)	3,065 (91.5)	
Yes	194 (16.2)	76 (14.4)	271 (15.5)	285 (8.5)	<0.001

Table 1. Characteristics of the study population included in the analyses of waterborne ingested nitrate: 1,869 cases of colorectal cancer (1,285 colon and 557 rectum) and 3,530 controls (Continued)

	Cancer cases				
	Colon *n* (%)	Rectum *n* (%)	Colorectal *n* (%)	Controls *n* (%)	*p* values[2]
Body mass index (Kg/m^2)[4]					
≤18.5–24.9	407 (31.7)	194 (34.8)	610 (32.7)	1,338 (37.9)	
25–29.9	585 (45.6)	243 (43.6)	840 (45.0)	1,482 (42.0)	
≥30	292 (22.7)	120 (21.5)	418 (22.4)	707 (20.1)	0.001
Energy intake (kcal/day)[4]					
<1,626	366 (31.7)	132 (26.0)	508 (30.2)	1,058 (33.4)	
>1,626–2,071	341 (29.6)	167 (32.9)	513 (30.4)	1,058 (33.3)	
>2,071	446 (38.7)	208 (41.0)	664 (39.4)	1,057 (33.3)	<0.001
Fiber intake (g/day)[4]					
<17	439 (38.1)	195 (38.5)	646 (38.3)	1,058 (33.4)	
17–23.5	373 (32.4)	156 (30.8)	537 (31.9)	1,058 (33.3)	
>23.5	341 (29.6)	156 (30.8)	502 (29.8)	1,057 (33.3)	0.002
Alcohol intake (g/day)[4]					
≤8	512 (44.4)	217 (42.8)	740 (43.9)	1,615 (50.9)	
>8	641 (55.6)	290 (57.2)	945 (56.1)	1,558 (49.1)	<0.001
Vitamin C (mg/day)[4]					
<117	415 (36.0)	208 (41.0)	634 (37.6)	1,058 (33.4)	
117–186	405 (35.1)	168 (33.1)	583 (34.6)	1,058 (33.3)	
>186	333 (28.9)	131 (25.8)	468 (27.8)	1,057 (33.3)	<0.001
Vitamin E (mg/day)[4]					
<8.5	420 (36.4)	177 (34.9)	603 (35.8)	1,058 (33.4)	
8.5–12.0	382 (33.1)	168 (33.1)	562 (33.4)	1,058 (33.3)	
>12.0	351 (30.4)	162 (32.0)	520 (30.9)	1,057 (33.3)	0.138
Red meat (g/day)[4]					
<20	321 (27.8)	117 (23.1)	444 (26.4)	1,058 (33.4)	
20–40	362 (31.4)	167 (32.9)	537 (31.9)	1,058 (33.3)	
>40	470 (40.8)	223 (44.0)	704 (41.8)	1,057 (33.3)	<0.001
Processed meat (g/day)[4]					
<17	355 (30.8)	132 (26.0)	498 (29.6)	1,058 (33.4)	
17–34	369 (32.0)	148 (29.2)	520 (30.9)	1,058 (33.3)	
>34	429 (37.2)	227 (44.8)	667 (39.6)	1,057 (33.3)	<0.001
Water intake (L/day)					
<0.9	389 (30.3)	185 (33.2)	584 (31.2)	1,181 (33.5)	
≥0.9–1.4	410 (31.9)	167 (30.0)	584 (31.2)	1,209 (34.2)	
>1.4	486 (37.8)	205 (36.8)	701 (37.5)	1,140 (32.3)	0.001

[1]Numbers of colon and rectum cases do not add 1,869 since 27 cases were undefined.
[2]*p values for Chi2 test* comparing controls versus CRC cases, calculated ignoring missing values in covariables.
[3]METs: Metabolic equivalents of task. Categories for physical activity were specific for each country. Cut offs for Italy are between parenthesis.
[4]Numbers do not add total cases and controls because of missing observations.
[5]Descriptive for women.

from administrative records of primary health care centers located within the hospitals' catchment areas in Spain, where universal health coverage is available. Potential participants were contacted telephonically on behalf of their family physician. For each control needed, five potential participants of similar age, and same sex and hospital catchment area were

selected. If contact with the first person of the list was not achieved (after at least five attempts at different times of the day), or if he/she refused to participate, the following person of the list was approached.[20] The study protocol was approved by the ethics committees of the participating institutions, and all participants signed an informed consent before recruitment.

Individual information and response rates

Study subjects were interviewed face-to-face by trained study personnel. Interviews were conducted in the hospitals (cases and hospital-based controls) and in primary health care facilities or nearby research centers (population-based controls). Questionnaires used are available online (http://mccspain.org). Data collected included sociodemographic characteristics; residential history from age 18 years to recruitment; water type consumed in each residence (municipal/bottled/well/other); amount of daily water intake (including water per-se, coffee, tea and other water-based beverages); smoking habits; history of gastric ulcer, diabetes, inflammatory bowel disease or Crohn's disease, use of non steroidal anti-inflammatory drugs (NSAIDs); oral contraceptive (OC) use, and hormonal replacement therapy (HRT); leisure physical activity since age 16 (Spain) or 15 years (Italy); family history of CRC, and information on the quality of the interview. Long-term exposure levels to trihalomethanes (THMs) in drinking water were available for the study population. Dietary information, corresponding to 2 years before recruitment, was collected using validated food frequency questionnaires (FFQs).[21,22] The FFQs included 140 (Spain) or 78 (Italy) food-items, and were administered during the interview in Italy (as part of the main questionnaire) or self-administered in Spain. Average response rates among cases were 58% in Spain (ranging from 33% to 80% among areas) and 95% in Italy, and among controls were 52% Spain (ranging from 30% to 68% among areas) and 95% in Italy. Average response rate for the FFQ in Spain was 88%. In total, 2,371 cases (1,905 Spain, 466 Italy) and 4,159 controls (3,590 Spain, 569 Italy) were interviewed.

Nitrate levels in municipal drinking water

We collected data for the municipalities covering 80% of total person-years in each area. We sent a standardized questionnaire to local authorities and water companies to ascertain current and historical nitrate measurements at the distribution system, and water source characteristics (surface/groundwater proportion). Monitoring levels for 2004–2010 were provided in Spain by the SINAC (*Sistema de Información Nacional de Aguas de Consumo*), and by the Regional Environmental Health Agency (Milan) and the Local Health Authority (Pordenone/Udine) in Italy. Measurements below the quantification limits (QL) (5% of measurements) were imputed half the QL value. If the QL value was missing, the measurement was imputed half of the most frequent QL reported (1.0 mg/L). More details on environmental data available are presented in Supporting Information (Table 1).

Nitrate levels in non-municipal drinking water

Data from the most consumed bottled water brands were available from previous reports in Spain[23] and Italy.[24] Nitrate levels in wells and springs outside the municipal water distribution system were measured in September 2013 in the area of León (Spain), where non-municipal well water consumption was the highest among the study areas (28% of controls in the longest residence). A total of 28 water samples were collected in 21 municipalities. The proportion of well water consumption in other areas ranged from 0.3% to 24% in the longest residence (33 years long, on average). These were considered as missing values given the lack of well water data in those areas.

Estimation of long-term nitrate levels in drinking water

We explored heterogeneity of nitrate levels within each municipality, by comparing the levels available for different sampling points, to identify water zones, defined as geographical areas supplied from a homogeneous water source and with similar nitrate levels. Most of the municipalities comprised only one water zone, and some of the municipalities (*e.g.*, Barcelona and Milan) had water zones already defined with different water sources. Long-term nitrate levels were estimated for 349 water zones, in total (Supporting Information Table 1). We calculated annual average by water zone using available measurements. For years without measurements, we back extrapolated the average of total measurements in the water zone back to 1940, as long as water source remained constant. Nitrate levels in ground water sources are usually higher than in surface sources.[25] Therefore, we used ground water percentage as a weight to calculate nitrate estimates when water source changed, assuming that levels increased proportionally to the percentage of ground water supplied. This assumption was evaluated for each water zone, and was applied uniformly in all municipalities where data was not sufficient to conduct this evaluation. In municipalities without nitrate measurements (covering 0.5% of the total person-years), we assigned the levels of neighboring municipalities supplied with similar surface/ground water proportion $\pm 10\%$. We defined a reliability score for each annual nitrate estimate, ranging from 0 (lowest reliability) to 2 (highest), that penalized estimates that were imputed, calculated based on few number of measurements, and more distant in time to an actual measurement. We used this score for sensitivity analyses.

Estimation of waterborne nitrate exposure

We linked nitrate levels with residential histories by year and municipality (or water zone) covering an exposure period from age 18 to 2 years before the interview ("adult life"), among cases and controls. Since more nitrate measurements were available in recent decades, our "main exposure period"

covered from 30 to 2 years before the interview. We also evaluated an exposure period from age 18 to 30 years ("early adult life"). We calculated average residential levels (mg/L as NO_3^-) and average waterborne ingested nitrate (mg/day) for each exposure period.

We calculated waterborne ingested nitrate according to amount and type of water consumed. We assigned residential levels when subjects reported tap water consumption. Published levels in bottled water brands were averaged using the sales frequency of each brand as a weight and were assigned when bottled water consumption was reported (6.1 mg/L in Spain and 3.8 mg/L in Italy). Levels from well water samples in León (range 0.5–93 mg/L) were assigned to well water consumers in this area, according to the postal code of wells' location. The annually assigned levels were averaged and multiplied by the daily water intake (mean ± SD = 1.4 ± 0.8 L/day in cases and 1.3 ± 0.9 L/day in controls). Water intakes above the 99th percentile (4 L/day), considered non plausible, were treated as missing values in the analyses.

To address the potential misclassification of the water type consumed (municipal/bottled) in recent residences, we calculated an alternative variable of waterborne ingested nitrate. We assumed that subjects reporting bottled water consumption and living during at least 10 years in the current (or previous) residence, consumed municipal water before the year 2000 and bottled water thereafter. This was assumed based on results from a subgroup with information on water type changes within residences ($n = 174$), showing that among 86% of subjects reporting bottled water consumption in the current residence, actually switched from municipal to bottled water after the year 2000. Similar calculations were done for Italy, using the cutoff at 1980 according to Italian data.

Estimation of dietary nutrients and nitrate

Data collected through FFQs were used to estimate the average daily intake of food groups and nutrients (vitamins C, E, and energy). Nutrients' contents were calculated using published food composition databases.[26,27] Dietary nitrate intake (mg/day) was estimated based on average intake of food items (g/day) and published nitrate content (mg/100 g) in food items including vegetables,[4] animal products and others.[28,29] Nitrate contents (mg/100 g) were calculated for 21 vegetables (including tubers), 13 fruits, 17 foods from animal sources (including red, white, processed meat and dairy products), 3 frequently consumed foodstuff (bread, rice and pasta) and 1 alcoholic beverage (beer). For these calculations "red meat" included: beef, lamb and pork meat. "Processed meat" included: bacon, hot dogs, smoked ham, Spanish cured ham and other cured sausages.

Statistical analyses

Subjects with nitrate exposure covering less than 70% of the last 30 years before the interview, and with unsatisfactory quality interview ($n = 24$) were excluded, leading to 1,869 cases and 3,530 controls analyzed. Nitrate exposure variables were categorized attempting to have subjects from different areas in all categories and high numbers in the reference. Odds ratios (OR) and 95% confidence intervals (CI) of CRC were calculated using mixed models with "area" as random effect. Basic models were adjusted for sex, age, study area and education. Potential confounders were explored overall and separately for men and women, including: smoking (never/ever), physical activity (measured in METs Metabolic equivalents of task/hour/week), body mass index (BMI), history of CRC in first degree relatives, NSAIDs use, OC use and HRT (in women), intake of energy, fiber, alcohol and endogenous nitrosation modulators (intake of vitamin C, vitamin E, red meat, processed meat and gastric ulcer history). Only variables that changed the risk estimates ≥10% were retained in the adjusted models.[11] In alternative analyses, models were adjusted for THM levels (residential and waterborne ingested) in the main exposure period. Missing values in categorical covariables were coded as another category. We evaluated the exposure-response relationship between waterborne nitrate exposure and CRC risk using generalized additive models (GAMs).

We stratified analyses of waterborne ingested nitrate by sex, cancer site, endogenous nitrosation modulators and other potential effect modifiers. Strata of quantitative variables (≤ or > median) were defined according to the distribution in controls. We compared the models with and without the interaction term using the likelihood ratio test, and p values less than 0.10 were considered indicative of multiplicative interaction. Stratified analyses by endogenous nitrosation factors were also conducted for men and women separately. We conducted several sensitivity analyses including the use of alternative variables of waterborne ingested nitrate in different exposure windows, and excluding exposure estimates (residential levels) with low reliability score (score value <0.50 $N = 1,077$). STATA version 12.0 (Stata Corp, College Station, TX) was used for all statistical analyses.

Results

Characteristics of the population analyzed are shown in Table 1. Family history of CRC, high BMI, high intake of energy, alcohol, red meat and processed meat were more frequent among cases (Chi^2 p values <0.05). The amount of water intake was also higher among cases (t test p values <0.05). Compared with the excluded, the subjects analyzed showed a higher proportion of controls, were younger, with lower physical activity, more frequent use of NSAIDs, and had lower (≤5 mg/L) or higher (≥10 mg/L) residential nitrate levels (Supporting Information Table 2).

On average (mean ± SD), this population had 3.3 ± 1.6 residences in adult life, and the time living in the most recent residence was 29.3 ± 14.9 years. The number of years (mean ± SD) with nitrate measurements available ranged from 4.0 ± 1.7 to 13.4 ± 1.5, among study areas. Nitrate measurements were available for 19% of the main exposure

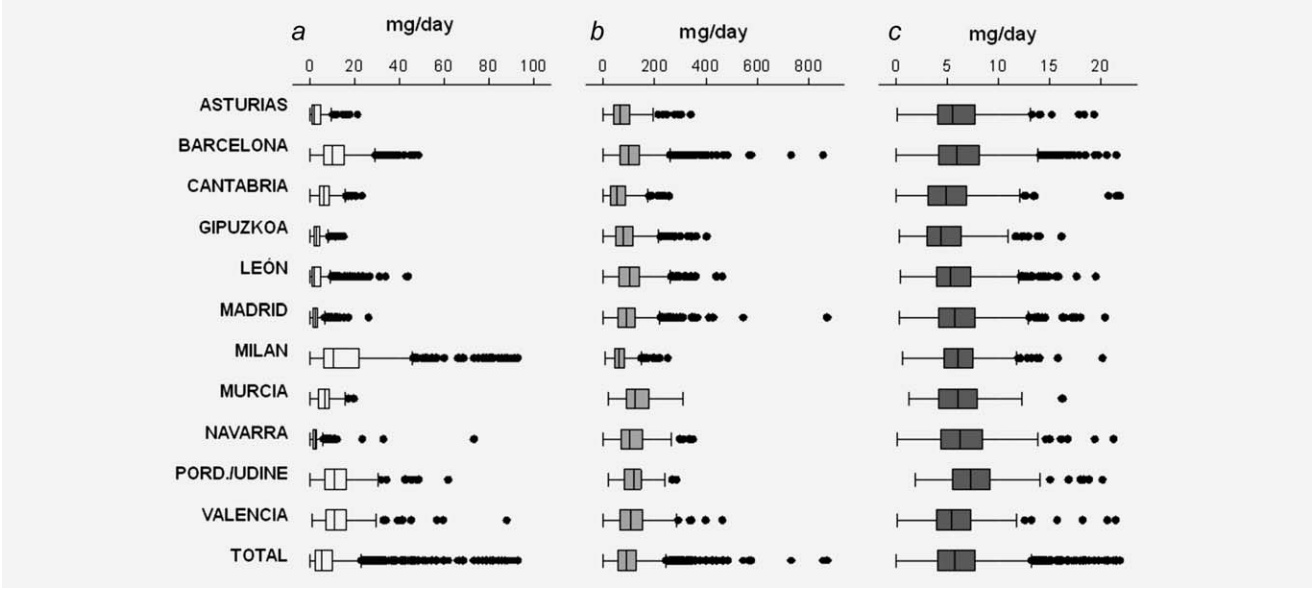

Figure 1. Average nitrate exposure levels among study areas. (*A*) Waterborne intake in the main exposure period (excluding intakes >105.8 mg/day, $n = 5$). (*B*) Dietary intake from vegetable sources (excluding intakes >1,000 mg/day, $n = 2$). (*C*) Dietary intake from animal sources (excluding intakes >22 mg/day, $n = 16$).

period (from 30 to 2 years before the interview), on average. Municipal water consumption was longer than bottled or well water consumption (19.2 ± 12.6 *vs.* 10.5 ± 16.5, and 0.8 ± 4.2 years, respectively), with differences among regions (Supporting Information Table 1).

Figure 1 shows average nitrate exposure levels through drinking water and diet during the main exposure period (from 30 to 2 years before the interview). Waterborne ingestion (mean ± SD) ranged from 3.4 ± 3.3 to 19.7 ± 22.6 mg/day, and residential levels ranged from 1.6 ± 0.9 to 30.0 ± 4.4 mg/L, among areas. The levels were similar in other exposure periods (results not shown). A high correlation was found between waterborne nitrate ingestion during the main exposure period and alternative exposure periods (*Spearman* correlation coefficients $r = 0.98$ with levels in adult life, and 0.91 with levels in early adult life). Dietary nitrate intake (mean ± SD) was 118 ± 72 mg/day (102 ± 70.5 mg/day from vegetables and 6.2 ± 3.3 mg/day from animal sources).

Table 2 shows the risk of CRC associated with waterborne ingested nitrate, overall and stratified by sex, for colon and rectum cancers sites. Adjusted ORs (95%CIs) of CRC for >10 versus ≤5 mg/day were 1.49 (1.24–1.78) overall, 1.50 (1.21–1.87) among men and 1.41 (1.04–1.91) among women. Interaction by sex was statistically significant for colorectal and colon, but not for rectal cancer. Results differed moderately by cancer site. The analyses of the alternative exposure periods led similar results, as well as the sensitivity analyses excluding subjects with low reliable score, or the subjects with less reliable interviews. Stratified analyses by time living in the current residence (≤15 years, >15–30 years and >30 years) also led similar results (not shown in tables). The ORs decreased slightly with additional adjustment for chloroform levels, while slightly increased after adjustment for brominated THMs (see Supporting Information Table 3).

Average residential nitrate levels were also associated with increased CRC (see Supporting Information Table 4), although the ORs were higher than those observed with waterborne ingested nitrate. These variables were moderately correlated, overall (*Spearman* correlation coefficient $r = 0.66$), but with wide differences among areas (*e.g.*, −0.04 in Madrid to 0.39 in León). In sensitivity analyses, areas with more than 10% of cases (Barcelona, León and Madrid) and Italian areas were alternatively excluded from the models. The ORs (95%CI) for the highest exposure category (>10 *vs.* <5 mg/L) decreased mostly after excluding Barcelona and Italian areas, but remained statistically significant. The ORs were higher among men versus women (interaction p values <0.001), and slightly higher for colon versus rectum tumors.

Figure 2 shows the GAMs for waterborne ingested nitrate. A small increase in CRC risk was found at ingested levels between 10 and 30 mg/day, among men and overall. At higher levels, the exposure-response curve was flat, with wide CIs. Area-specific GAMs showed heterogeneous exposure-response curves between areas (Supporting Information Fig. 1).

Table 3 shows stratified analyses by dietary endogenous nitrosation factors and fiber intake, overall and by cancer site. High ORs (95%CI) were found in the groups with highest waterborne ingested nitrate and highest red meat intake, particularly among men: 1.71 (1.30, 2.26) (see results by sex in Supporting Information Table 5). Results for processed meat were similar to results for red meat, overall (not shown in tables). Inverse ORs (95%CI) of CRC were found among the groups with low ingested nitrate and high vitamin E

Table 2. Colorectal cancer risk associated with waterborne nitrate ingestion during the main exposure period in all population ($n = 5{,}399$) and stratified by cancer site and sex

Mean nitrate ingestion	≤5 mg/day			>5–10 mg/day			>10 mg/day		
	Cases	Contr.	OR[1] (95%CI)	Cases	Contr.	OR[1] (95%CI)	Cases	Contr.	OR[1] (95%CI)
Cancer site									
Colorectal[2]									
All	778	1,899	1.00 (ref.)	447	803	1.17 (0.98, 1.38)	644	828	1.49 (1.24, 1.78)
Men	498	918	1.00 (ref.)	289	454	1.16 (0.94, 1.44)	397	468	1.50 (1.21, 1.87)
Women	280	981	1.00 (ref.)	158	349	1.20 (0.90, 1.58)	247	360	1.41 (1.04, 1.91)
Colon[2]									
All	527	1,899	1.00 (ref.)	324	803	1.28 (1.06, 1.55)	434	828	1.52 (1.24, 1.86)
Men	322	918	1.00 (ref.)	202	454	1.26 (0.99, 1.61)	260	468	1.51 (1.17, 1.94)
Women	205	981	1.00 (ref.)	122	349	1.33 (0.97, 1.80)	174	360	1.46 (1.04, 2.05)
Rectum[2]									
All	244	1,899	1.00 (ref.)	110	803	0.93 (0.70, 1.23)	203	828	1.62 (1.23, 2.14)
Men	169	918	1.00 (ref.)	80	454	0.94 (0.68, 1.28)	133	468	1.55 (1.16, 2.08)
Women	75	981	1.00 (ref.)	30	349	0.87 (0.52, 1.45)	70	360	1.49 (0.89, 2.48)

[1]Odds ratios (OR) and 95% confidence intervals (CI). Results of mixed models with "area" as random effect, adjusted for: sex, age, education, body mass index, physical activity, non-steroidal anti-inflammatories use, family history of colorectal cancer and intake of energy. Analyses for women were also adjusted for oral contraceptives use.
[2]Interaction *p values* by sex = *0.01* for colorectal, *0.05* for colon and *0.15* for rectal cancer.

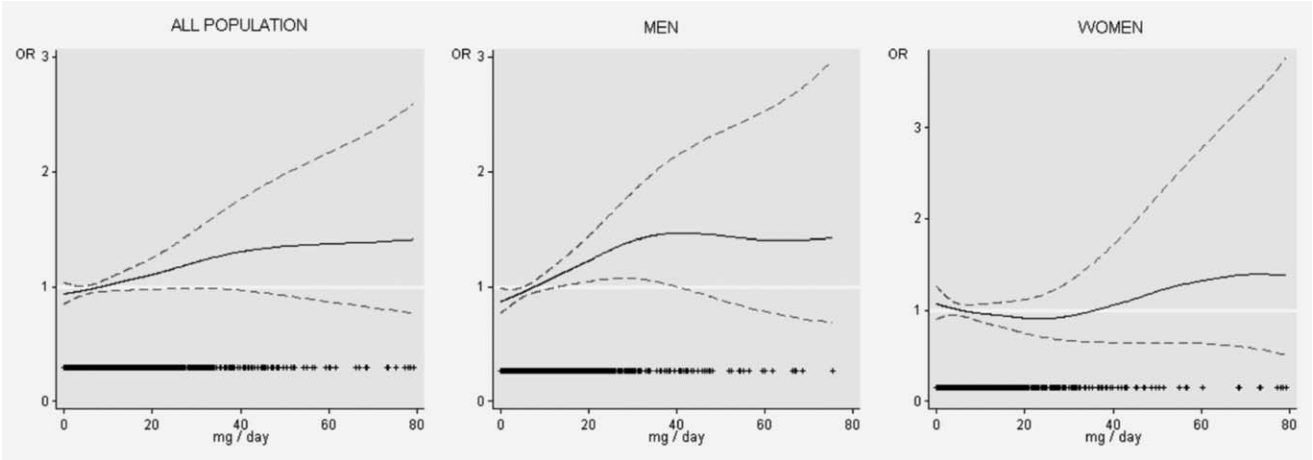

Figure 2. Exposure-response relationship between average waterborne ingested nitrate (mg/day) during the main exposure period and colorectal cancer risk. Generalized additive models (GAMs) adjusted for study area, sex, age, education, physical activity, body mass index, use of non-steroidal anti-inflammatory drugs, family history of colorectal cancer and energy intake. Subjects with ingestion levels >80 mg/day ($n = 21$) were excluded from these analyses.

(0.73, 0.59–0.89), and high fiber intake (0.65, 0.53–0.80). Results by cancer site showed similar results for colon cancer, but inverse ORs with vitamin C and fiber were found for rectum cancer. Stratified results did not differ by diabetes diagnosis, or smoking (not shown).

Table 4 shows the risk of CRC (overall and by cancer site) associated with dietary ingested nitrate. An association (OR, 95%CI) with nitrate intake from animal sources was found for rectal cancer (1.55, 1.17–2.05) while no association was observed for colon cancer (1.06, 0.87–1.30). Ingestion from total diet or vegetables led to null or inverse associations with CIs around the null value for both cancer sites. Waterborne and dietary ingested nitrate were poorly correlated (*Spearman* correlation coefficient for nitrate from total diet: 0.07, vegetables: 0.06 and animal sources: 0.07). The adjustment of waterborne ingested nitrate analyses' for dietary nitrate intake (through different sources) did not change the main results (results not shown).

Discussion

Results of this large case–control study suggest a positive association between CRC risk and long-term exposure to nitrate in drinking water, at levels below 50 mg/L of NO_3^-, particularly in subgroups of the population, such as men and

Table 3. Colorectal cancer risk associated with waterborne ingested nitrate levels in the main exposure period. Joint effect with endogenous nitrosation factors and other dietary covariables in all population and by cancer site

Covariable Intake	Nitrate intake mg/day	Colorectal cancer ($n = 4{,}858$[1])		Colon cancer ($n = 4{,}326$[1])		Rectal cancer ($n = 3{,}680$[1])		Controls
		Cases	OR[2] (95%CI)	Cases	OR[2] (95%CI)	Cases	OR[2] (95%CI)	
Red meat								
≤29 g/day	≤4.4	296	1.00 (ref.)	209	1.00 (ref.)	85	1.00 (ref.)	842
	>4.4	380	1.09 (0.88–1.35)	265	1.12 (0.88, 1.44)	107	1.08 (0.76, 1.55)	744
>29 g/day	≤4.4	349	1.24 (1.02–1.51)	221	1.17 (0.93, 1.47)	124	1.49 (1.09, 2.02)	742
	>4.4	660	1.56 (1.26–1.94)	458	1.66 (1.30, 2.12)	191	1.57 (1.11, 2.21)	845
Interaction p values			0.27		0.12		0.90	
Vitamin C								
≤137 mg/day	≤4.4	343	1.00 (ref.)	218	1.00 (ref.)	121	1.00 (ref.)	766
	>4.4	589	1.20 (0.98–1.47)	409	1.36 (1.08, 1.71)	168	0.99 (0.73, 1.36)	820
>137 mg/day	≤4.4	302	0.83 (0.68–1.01)	212	0.92 (0.73, 1.15)	88	0.67 (0.49, 0.91)	818
	>4.4	451	0.95 (0.77–1.18)	314	1.09 (0.86, 1.38)	130	0.77 (0.56, 1.07)	769
Interaction p values			0.73		0.35		0.45	
Vitamin E								
≤10 mg/day	≤4.4	355	1.00 (ref.)	238	1.00 (ref.)	112	1.00 (ref.)	806
	>4.4	531	1.15 (0.94, 1.42)	373	1.26 (1.00, 1.59)	149	1.03 (0.75, 1.43)	780
>10 mg/day	≤4.4	290	0.73 (0.59, 0.89)	192	0.73 (0.57, 0.93)	97	0.75 (0.54, 1.04)	778
	>4.4	509	0.87 (0.70, 1.09)	350	0.94 (0.73, 1.21)	149	0.81 (0.57, 1.15)	809
Interaction p values			0.74		0.89		0.85	
Fiber								
≤20 g/day	≤4.4	344	1.00 (ref.)	224	1.00 (ref.)	115	1.00 (ref.)	741
	>4.4	595	1.13 (0.92–1.38)	422	1.28 (1.01, 1.61)	159	0.90 (0.66, 1.24)	845
>20 g/day	≤4.4	301	0.65 (0.53–0.80)	206	0.71 (0.56, 0.89)	94	0.55 (0.40, 0.76)	843
	>4.4	445	0.84 (0.68–1.04)	301	0.92 (0.73, 1.18)	139	0.72 (0.52, 1.00)	744
Interaction p values			0.32		0.88		0.07	

[1]Numbers do not add 5,399 due to missing values in dietary variables and cancer site.
[2]Odds ratios (OR) and 95% confidence intervals (CI). Results of mixed models with "area" as random effect adjusted for: sex, age, education, physical activity, non-steroidal anti-inflammatory drugs use, family history of colorectal cancer and energy intake.

Table 4. Dietary ingested nitrate and colorectal cancer risk stratified by cancer site among population with available dietary information ($n = 4{,}858$)

Ingested nitrate from:	Median intake (mg/day)	Colorectal cancer		Colon cancer		Rectal cancer		Controls
		Cases[1]	OR[2] (95% CI)	Cases	OR[2] (95% CI)	Cases	OR[2] (95% CI)	
Animal sources								
<4.5 mg/day	3.1	473	1.00 (ref.)	352	1.00 (ref.)	112	1.00 (ref.)	1,058
4.5–6.8 mg/day	5.6	578	1.15 (0.98, 1.35)	378	1.03 (0.86, 1.24)	191	1.59 (1.22, 2.06)	1,058
>6.8 mg/day	9.4	634	1.16 (0.98, 1.38)	423	1.06 (0.87, 1.30)	204	1.55 (1.17, 2.05)	1,057
Continuous			1.03 (1.01, 1.05)		1.02 (1.00, 1.04)		1.04 (1.01, 1.07)	
Vegetable sources[3]								
<68 mg/day	42	597	1.00 (ref.)	392	1.00 (ref.)	194	1.00 (ref.)	1,058
68–118 mg/day	92	575	0.99 (0.85, 1.16)	397	1.04 (0.87, 1.24)	169	0.91 (0.71, 1.16)	1,058
>118 mg/day	179	513	0.83 (0.70, 0.99)	364	0.89 (0.73, 1.08)	144	0.75 (0.57, 0.99)	1,057
Continuous			1.00 (0.99, 1.00)		1.00 (0.99, 1.00)		1.00 (0.99, 1.00)	
Total diet[3]								
<83 mg/day	59	594	1.00 (ref.)	388	1.00 (ref.)	195	1.00 (ref.)	1,058
83–133 mg/day	108	564	0.97 (0.83, 1.14)	394	1.04 (0.87, 1.24)	161	0.85 (0.66, 1.08)	1,058
>133 mg/day	176	527	0.84 (0.70, 1.00)	371	0.90 (0.74, 1.10)	151	0.76 (0.58, 1.00)	1,057
Continuous			1.00 (0.99, 1.00)		1.00 (0.99, 1.00)		1.00 (0.99, 1.00)	

[1]This column does not add to total number of cancer cases since 46 were undefined site.
[2]Odds ratios (OR) and 95% confidence intervals (CI). Results of mixed models with "area" as random effect adjusted for sex, age, education, physical activity, non-steroidal anti-inflammatory drugs use, family history of colorectal cancer, body mass index and intake energy.
[3]Results are also adjusted for fiber intake.

subjects with high red meat intake. The associations slightly differed for colon and rectal cancer. A positive association was found between rectal cancer risk and nitrate intake from animal sources, but an inverse association is suggested with intake from vegetables.

This is one of the few studies evaluating CRC risk and nitrate exposure through drinking water. Our results are comparable to previous case–control studies from the United States, although those studies evaluated residential, but not ingested nitrate, at higher levels than those observed in our study. A 2.9-fold increased risk of proximal colon cancer for NO_3-N residential levels $\geq$10 mg/L (44 mg/L of NO_3^-) versus $<$0.5 mg/L has been reported.[30] Increased risk of colon cancer was found among subjects with residential NO_3-N levels $>$5 mg/L for $>$10 years and low vitamin C intake or high meat intake.[16] Other available studies had ecologic design or ignored endogenous nitrosation factors and individual water consumption data,[31,32] thus are not totally comparable to our study.

Dietary ingested nitrate levels in this study were similar to those observed in other western countries.[33] Our results are consistent with a cohort study[15] that found higher colon cancer risk with ingested nitrate from animal sources. Results from other studies on CRC and dietary nitrate, nitrite or NOCs are heterogeneous,[5,6,13,14] and most of them did not evaluate ingestion from different dietary sources. In contrast to results for animal-derived nitrate, inverse associations were found for high nitrate intake from vegetables. These results may not be confounded by the protective effect of fiber, since the analyses were adjusted for this variable. The presence of endogenous nitrosation inhibitors in vegetables and hypothesized beneficial effects of nitrate from vegetables[34] may partly explain these findings.

Our results suggest an interaction between waterborne nitrate exposure and sex for CRC and colon cancer, but not for rectal cancer. The associations were higher among men, similarly to other exposures such as dietary factors.[35] This may partly be attributed to the protective effect of estrogens and other hormonal factors.[36] We found higher associations in groups with high red meat intake. These results were consistent with previous studies that evaluated other cancer types associated with nitrate or nitrite exposure.[33,37] Although the interaction with red meat was not statistically significant, is plausible, because red meat contains amines, amides, and heme iron which may increase endogenous formation of NOCs.[38] Information on heme iron intake was not available in this study, but should be accounted to evaluate the interaction with red meat in future analysis.[39] In contrast, inverse associations were found in groups of high vitamin E or fiber intake. Vitamins E and C inhibit endogenous nitrosation, and a protective effect is biologically plausible. The combined intake of vitamins C and E showed similar effects to those shown for each vitamin. The protective effect of fiber was also expected, based on previous evidence on fiber intake and CRC risk.[40] Apart from endogenous nitrosation, changes in gastrointestinal microbiota,[41] and genetic variants of CYP2E1 (involved in NOCs' bio-activation),[14] may also play a role in carcinogenesis of ingested nitrate and should be explored in future analyses.

Confounding by other water contaminants such as THMs[42] was a concern. In this study, estimates of THMs intake were available, and were evaluated as potential confounders. Associations decreased slightly after adjusting for chloroform, and increased slightly after adjusting for brominated THMs or total THMs, but the differences were not statistically significant. The potential interaction of these frequent water contaminants requires further evaluation, since contradictory effects are suggested for chlorinated versus brominated THMs. Other water contaminants showed levels around or below the QL in our study areas,[23] and are not likely to be relevant confounders in the context of this study.

Although different response rates between study areas and relatively low rates among controls may be a limitation, nonparticipation is unlikely related to nitrate exposure. Potential exposure measurement error is a limitation, since nitrate measurements in drinking water were only available in recent years. Missing historical levels were estimated based on recent measurements and were assumed to remain stable over time, depending on groundwater percentages. This assumption may introduce measurement error, particularly for long-term periods (*e.g.*, adult life). Nitrate levels may differ widely between groundwater sources according to the depth of wells, and may change in time according to factors other than water source (*e.g.*, agricultural practices). Such information was not collected, since the questionnaire was not originally designed to estimate historical nitrate levels, and was not available in official reports. However, we analyzed the municipalities with longest nitrate records: Llíria (Valencia) and Donostia (Gipuzkoa), and no significant changes were found in nitrate levels over 17 years. The levels estimated for a 30-year period would be sufficient to evaluate the association with CRC risk, among this population. Additionally, we applied several strategies to address the potential exposure measurement error: we analyzed only the population with exposure information available for $\geq$70% of the main exposure period (30 to 2 years before the interview). We performed sensitivity analyses excluding less reliable exposure estimates, obtaining similar results to those shown in Table 2. We analyzed three different exposure periods, but results for adult life and early adult life are limited because are based on estimates with low reliability. In addition, nitrate estimates from different exposure periods were highly correlated. Studies in other settings, with larger availability of historical environmental data, are needed to increase the current evidence on waterborne nitrate exposure and CRC risk.

Since dietary information was collected with a FFQ, recall bias may not be totally ruled out in the analyses for dietary nitrate. The results for dietary nitrate intake may not be extrapolated for long-term periods, since dietary information corresponded to the last 2 years previous to recruitment. Estimates of dietary nitrate are prone to measurement error since we used the same nitrate contents in food products,

regardless of potential country-specific levels. However, the database used is valid for all European countries, and includes specific Spanish and Italian measurements.[4] Data on relevant vegetable sources of nitrate was not completely available, and data on storage and processing (*i.e.*, washing, peeling and cooking) was not collected, which also may introduce error in calculations of nitrate intake from vegetables. Finally, dietary nitrite intake was not available, but this would not be a major limitation since the main exposure route expected is through endogenous nitrate reduction.[8]

The wide differences on nitrate levels between study areas, and the low variability within areas hampered the statistical analyses. We applied different approaches for all-area combined analyses, including unconditional logistic regression, GAMs, and meta-smoothing analyses[43] (previously used in multicentric studies on air pollution). Mixed models, with area as random effect were finally applied given the heterogeneity of results between study areas. This heterogeneity is a limitation, and is probably related to other environmental or individual factors that were not evaluated in this study. The results of mixed models differed slightly from results of the GAMs, particularly among women. Results among women may be less robust due to the smaller sample size, compared with men. Results of meta-smoothing analyses are not shown, because were equivalent to results of the GAMs.

A main strength of this study was the availability of detailed individual information, allowing the assessment of several potential confounders and effect modifiers, including other frequent water contaminants (THMs) and endogenous nitrosation factors. In addition, the FFQ information enabled us to assess nitrate exposure through different dietary sources. Nitrate measurements in non municipal water (wells) were measured and included in the exposure assessment for the area with the highest consumption of this water type (León). Finally, the main results were robust, as were replicated using different approaches for statistical analysis.

Conclusions

Overall, effects of nitrate exposure differed by exposure source (water, vegetables and animal dietary sources). A positive association is suggested between CRC risk and long-term exposure to nitrate in drinking water at levels below the European regulatory limit, particularly among subjects with other risk factors. Dietary nitrate from animal sources increased rectal cancer risk, but high intake from vegetables seems to decrease it. Further research is required to confirm these findings.

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