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ABSTRACT

Background: Ingested nitrate leads to endogenous formation of N-nitroso compounds that are breast carcinogens in animals, but human evidence is limited.

Objective: We evaluated ingested nitrate as a risk factor for breast cancer (BC) in a multicase-control study.

Methods: Hospital-based incident BC cases and population-based controls were recruited in eight Spanish regions in 2008-2013, providing residential and water consumption from age 18 years, and information on known BC risk factors. Long-term nitrate levels (1940-2010) were estimated and linked with residential histories and water consumption to calculate waterborne ingested nitrate (mg/day). Dietary ingested nitrate (mg/day) was calculated using food frequency questionnaires and published dietary nitrate contents. Interactions with endogenous nitrosation factors and other variables were evaluated. A total of 1245 cases and 1520 controls were included in the statistical analysis.

Results: Average ±SD waterborne ingested nitrate ranged from 2.9 ±1.9 to 13.5 ±7.5 mg/day and dietary ingested nitrate ranged from 88.5 ±48.7 to 154 ±87.8 mg/day, among regions. Waterborne ingested nitrate was not associated with BC overall, but among postmenopausal women, those with both high nitrate (>6 vs. <2.6 mg/day) and red meat intake (≥20 vs. <20 g/day) were more likely to be cases than women with low nitrate and low red meat intake (adjusted odds ratio= 1.64; 95% confidence interval: 1.08, 2.49; overall interaction p value= 0.17). No association was found with dietary nitrate.

Conclusions: Waterborne ingested nitrate was associated with BC only among postmenopausal women with high red meat consumption. Dietary nitrate was not associated with BC, regardless of the animal or vegetable source, or menopausal status.

INTRODUCTION

Breast cancer (BC) is the first cause of cancer mortality and the most common cancer among women worldwide. In Spain, 25,215 new cases are annually diagnosed (Ferlay et al. 2013) with increasing incidence rates during the last decades (Pollán et al. 2009). Several risk factors have been identified, including sex, age, nulliparity, short breastfeeding, menstrual and reproductive history, high body mass index (particularly in post-menopausal women), physical inactivity, high alcohol or energy intake, use of drugs with estrogenic action, exposure to ionizing radiation, specific genetic factors, family history of BC, previous diagnosis of non-malignant breast diseases and high mamographic density (Hankinson et al. 2004; Romieu et al. 2015; Stewart 2014). Established risk factors explain around 50% of the incidence variation of this tumor, and other environmental exposures may partly explain the remaining variation (Brody et al. 2007).

Nitrate is a frequent contaminant in drinking water worldwide, related to excessive fertilizers´ use or sewage (Wakida and Lerner 2005). Humans are exposed to nitrate through diet and drinking water ingestion. The maximum contaminant level in drinking water (50 mg/L as nitrate ion NO_3^- or 10 mg/L of nitrate-N) (EU 1998; WHO 2008) was established to prevent acute health effects in children (methemoglobinemia), but the effects of long-term exposure to lower levels, including cancer risk, are not well established (Ward et al. 2005).

Ingested nitrate is classified as a probable human carcinogen in conditions of endogenous nitrosation (IARC 2010). This process involves the conversion of nitrate into nitrite, and the synthesis of N-nitroso compounds (NOCs) in the gastrointestinal tract. The intake of

antioxidant vitamins and the use of non-steroidal anti-inflammatory drugs (NSAIDs) inhibit endogenous nitrosation, while meat intake, and inflammatory gastrointestinal conditions promote it (Ward et al. 2005). NOCs are potent carcinogens for several animal species (Lijinsky et al. 1992). Some NOCs, like the N-methyl-N-nitrosourea (MNU), are used to induce BC in experimental animal studies, and young rats exposed to MNU were more susceptible to develop breast tumors (Tsubura et al. 2011). In cellular studies, low doses of nitrite and nitrate were able to mimic estradiol and activated estrogen receptors, suggesting a potential role of these anions in breast cancer´s etiology or progression (Veselik et al. 2008).

Despite the evidence in animals, few epidemiologic studies have evaluated the association between nitrate exposure or its derivatives and BC. Relevant available studies were conducted in the United States of America (Brody et al. 2006; Weyer et al. 2001), and did not find associations between waterborne or dietary ingested nitrate and BC. A recent cohort study of postmenopausal women in the US reported that BC was increased in the highest versus lowest quintile of water nitrate intake among women who also had folate ingestion of ≥400 μg/day, but did not find any association with dietary nitrate (Inoue-Choi et al. 2012). The authors of previous studies attributed their null associations to limitations in the exposure assessment (i.e. lack of data on water daily intake), the co-existence of antioxidants (i.e. vitamin C) in main dietary sources of nitrate (vegetables), and the lack of evaluation of nitrate intake from specific dietary sources such as animal foods and processed meat. In summary, human evidence relating nitrate exposure and BC is limited and inconclusive. Studies evaluating different exposure windows, including individual

water consumption information, endogenous nitrosation factors and other covariables, are required to enhance the available evidence.

We aimed to evaluate ingested nitrate through drinking water and diet as a risk factor for breast cancer (BC) in a population-based Multi Case-Control Study conducted in Spain (MCC-Spain).

METHODS

Study design and population

This study is part of the MCC-Spain study, aimed to evaluate the influence of environmental exposures on common tumors in Spain (e.g. female breast or colorectal). Study population was recruited between 2008 and 2013 in eight Spanish provinces (see Table 1). Cases were identified shortly after the diagnosis (average: 3.2 months, SD 4.2) through an active search by periodical visits to the collaborating hospital departments (i.e. gynecology, oncology, general surgery, radiotherapy, and pathology departments). Participant hospitals were the reference centers for oncologic diseases in each study area. Only incident cases diagnosed within the recruitment period, without malignant BC history, aged between 20 and 85 years-old, residing in the hospitals´ catchment areas for at least 6 months prior to recruitment, and being able to answer the epidemiological questionnaire (Castaño-Vinyals et al. 2015) were included. All cases had histological confirmation, and included all malignant BC (International Classification of Diseases 10th Revision [ICD-10]: C50), and frequent in situ breast cancers (ICD-10:D05.1, D05.7). Population-based controls were frequency-matched to cases by age, sex and region, ensuring to have at least one control of the same sex and 5-year interval age for each case.

Eligible controls were randomly selected from administrative records of primary care health centers located within hospitals' catchment areas. For each control needed, five potential participants of similar age, sex and hospital catchment area were randomly selected from the lists of general practitioners. If contact with the first person of this list was not achieved (after at least five tries at different times of the day), or if he/she refused to participate, the following person of the list was approached. The study protocol was approved by the ethical review board from each participating center and women signed an informed consent before recruitment.

Questionnaires and response rates

A structured computerized questionnaire was administered by trained personnel in face-to-face interviews (http://www.mccspain.org). Collected data included: a) Sociodemographic characteristics; b) Lifetime residential history; c) The water type consumed in each residence (municipal/bottled/well/other); d) The amount of water intake at home, including water per-se, coffee, tea and other water-based beverages; e) Smoking habits; f) History of gastric ulcer and use of NSAIDs; g) Gynecologic and reproductive history; h) Use of oral contraceptives (OC), or hormonal replacement therapy (HRT); and g) Physical activity. Anthropometric measurements were self-reported (weight, height) or measured (waist and hip circumference) during the interview. Histological type and estrogen receptors data were available for cases. Average response rates differed among regions and were 71% among cases and 53% among controls, overall (Castaño-Vinyals et al. 2015). In total, 1585 cases and 1822 controls were recruited and answered the questionnaire.

Dietary information, corresponding to one year previous to recruitment, or previous to diagnosis among cases, was collected using a validated food frequency questionnaire (FFQ) (Martin-Moreno et al. 1993). The FFQ comprised 140 food items, including regional Spanish products, and was either administered during the interview or self-administered and returned by mail. Instructions to complete the FFQ were provided during the interview. The FFQ was used to estimate the average daily intake of vegetables, fruits, meat, dairy products and alcoholic beverages.

Dietary nitrate and nutrient estimates

Published food composition tables (Farran et al. 2008) were used to calculate the daily intake of energy and nutrients (vitamins C, D, E and folate). Dietary nitrate intake (mg/day) was estimated based on average intake of food items (g/day) and published nitrate content (mg/100g) in food items including vegetables (EFSA 2008), animal products, and others (Griesenbeck et al. 2009; Jakszyn et al. 2004). Nitrate contents were assigned to the following food items: 21 vegetables (including tubers), 13 fruits, 17 animal sources (including red, white, processed meat and dairy products), frequently consumed foodstuff (bread, rice, and pasta), and one alcoholic beverage (beer). For calculations, “red meat” included: beef, lamb and pork meat; “Processed meat” included: bacon, hot dogs, smoked ham, Spanish cured ham and other cured sausages.

Nitrate levels in drinking water

We collected environmental data from municipalities covering 80% of person-years in each area. We sent a standardized questionnaire to local authorities and water companies, ascertaining current and historical nitrate measurements in water of municipal distribution

systems, and water source characteristics (surface/groundwater proportion). Monitoring levels (2004-2010) were provided by the SINAC (*Sistema de Información Nacional en Aguas de Consumo*). Measurements below the quantification limits (QL) (5% of measurements) were imputed half of the QL value. If the QL was missing, the measurement was imputed half of the most frequent QL reported (1.0 mg/L).

We measured nitrate levels in samples of the most consumed bottled water brands in Spain (Espejo-Herrera et al. 2013). Nitrate levels in wells and springs, not covered by the municipal water distribution system, were measured in September 2013 (unpublished data). A total of 28 water samples were collected in 21 municipalities of León region, where non-municipal water consumption was the highest among our study areas (26% of controls in the longest residence).

Estimation of long-term nitrate levels in drinking water

We calculated annual average nitrate levels back to 1940 by water zone (defined as a geographical area supplied by water with a homogeneous source and quality), that usually corresponded to municipality. We calculated annual averages based on available nitrate measurements. For years without measurements, we assigned the averaged total measurements available in the water zone, as long as water source remained constant. In case of changes in water source, the ground water percentage was used as a weight to modulate the estimations, assuming that nitrate levels were higher at higher ground water proportions. In municipalities without any nitrate measurement (covering 0.5% of the total person-years), we imputed the levels of neighboring municipalities supplied with similar ground water proportion ± 10%.

Individual exposure variables

We linked nitrate levels in drinking water (measured and imputed) and residential histories by year and municipality covering the exposure window from age 18 years to 2 years before the study interview (from now referred as "adult life" or "long-term exposure"). To calculate waterborne ingested nitrate (mg/day), we assigned nitrate levels (mg/L NO_3^-) in drinking water year-by-year according to the water type consumed. Nitrate levels in municipal water (residential levels) were assigned for tap water consumption. Levels in the sampled bottled waters were averaged using the sales frequency of each brand as a weight. This weighted average (6.1 mg/L of NO_3^-) was assigned when drinking bottled water consumption was reported. Levels in well water samples from León (range: 0.5- 93 mg/L) were assigned to women reporting well water consumption in this area, according to the postal code of the residence. Nitrate levels in well water were not available for other areas, and waterborne ingested nitrate was considered missing for years when well water consumption was reported among women from those areas (range: 0.6% to 8.2% of controls in the longest residence). The annual nitrate estimates were averaged and multiplied by the average daily water intake at home (1.3±0.7 L/day in cases, 1.2±0.7 L/day in controls). Water intakes above the 99th percentile (4L/day), considered non plausible, were treated as missing values in the analyses. We also calculated the average waterborne ingested nitrate in two alternative exposure periods: from 15 to 2 years before the interview ("recent" exposure), and from age 18 to 30 years ("early adulthood" exposure).

In a subset from Barcelona with information on water type changes within residences, 86% of subjects reporting bottled water consumption in the last residence, actually switched

from municipal to bottled water after the year 2000. Potential misclassification of the water type consumed (municipal/bottled), particularly in recent residences was a concern. To address this, we calculated an alternative variable for waterborne ingested nitrate in adult life. We assumed that women reporting bottled water consumption and living during at least 10 years in the last residence (or in the previous one), actually consumed municipal water before the year 2000, and bottled water thereafter.

Statistical analyses

The population analyzed (1245 cases, 1520 controls) included women with data on waterborne ingested nitrate covering at least 70% of the main exposure period (from age 18 to 2 years before the interview), and data on daily water intake. We estimated odds ratios (OR) and 95% confidence intervals (CI) of BC for categorized nitrate intake using unconditional logistic regression. Categories of exposure (quartiles or tertiles) were specifically defined for pre and post-menopausal women, according to the distribution in controls. Basic models were adjusted for age (continuous), study area, and education (3 categories: primary or less, high school, and university or more). Several potential confounders were explored separately for pre and post-menopausal women, including: smoking (yes/no 5 years before recruitment), average leisure physical activity since age 16 to 2 years before the interview (measured in metabolic equivalents of task (METS)/hour/week), body mass index (BMI), family history of malignant BC in any blood relative (yes/no), NSAIDs use (yes/no), age at menarche, age at menopause (continuous variables in years and categorized). Menopause and age at menopause were defined according to the date of the last regular menstrual period. Age at first birth, nulliparity

(yes/no), parity (number of births), total months of breastfeeding (categorized), OC and HRT use (never/ever), intake of alcohol (no/yes at age 30 years), intake of energy and folate (tertiles), and endogenous nitrosation modulators (intake of vitamin C, vitamin E, red meat, and processed meat) were also explored as potential confounders. Only established BC risk factors (Stewart 2014), and variables that changed the risk estimates >10% were included in the adjustment (age, study area, education, BMI, family history of BC, age at first birth, use of OC, energy intake and age at menopause for postmenopausal women). For each model covariate, missing data in categorical variables were classified as a separate category in multivariate analyses. Trend p-values were derived from a likelihood ratio test (LRT) comparing a model with the categorical nitrate variable as an ordinal variable (0, 1, 2), with a model that excluded the variable.

We used generalized additive models (GAMs) to evaluate the exposure-response relationship between waterborne nitrate intake and BC by study area. We stratified analyses for waterborne ingested nitrate by relevant covariates, including endogenous nitrosation factors (intake of vitamin C, vitamin E, red meat, and processed meat), folate intake and smoking. Strata of continuous variables were defined according to the distribution in controls (≤ or > median). We compared the multivariate models with and without the interaction term using a LRT, and p values <0.10 were considered indicative of multiplicative interaction. Analyses by histological type (ductal ICD-10: C50, and other in situ tumors ICD-10: D05.1, D05.7), and estrogen receptor (ER) status were also conducted. In sensitivity analyses, we used the alternative variables of waterborne ingested nitrate. We also excluded women with missing data in covariables, and women with missing or lower interview quality. Interview quality was assessed by the interviewers as unsatisfactory,

questionable, reliable or high quality interview, based on the completeness of the information provided. All statistical analyses were performed using STATA version 12.0 (Stata Corp, College Station, TX).

RESULTS

General characteristics of the study population are shown in table 1. Compared to controls, cases showed higher frequency of family history of BC, age at menopause >50 years, age at first birth >30 years, higher intake of energy, red meat, processed meat, lower intake of vitamin C and nulliparity, with statistically significant differences (p value <0.05 in Chi^2 test). Among women analyzed, 24.6% (N=679) were premenopausal and 75.4% (N=2086) postmenopausal. Women with nitrate levels in drinking water assigned for <70% of the residential history in adult life, and those without information on daily water intake were excluded from the analyses. In comparison to the excluded, women analyzed showed a higher proportion of controls (55% vs. 47%), and post-menopausal women (75.4% vs. 63.9%), were older, showed a lower proportion of university education, nulliparity, and lower intake of vitamins C and E, while levels of waterborne ingested nitrate were similar (see Supplemental Material, Table S1).

Figure 1 shows average ingested nitrate levels in adult life, among cases and controls. Waterborne ingested levels (mean± SD) ranged from 2.9 ±1.9 to 13.5±7.5 mg/day, among areas (Figure 1A), and were higher among post vs. premenopausal women (6.74 ±7.1 vs. 5.12 ±5.6 mg/day p value= <0.001 for U-Mann Whitney test). Ingested levels during alternative exposure periods (from 15 to 2 years before study interview and from age 18 to 30 years) were similar to levels presented in Figure 1A (results not shown). Dietary

ingested levels (mean ±SD) ranged from 88.5 ±48.7 to 154 ±87.8 mg/day, among areas (Figure 1B), and were higher among post vs. premenopausal women (129.0 ±86.2 vs. 109.7 ±62.1 mg/day *p* value <0.001 for T test). On average, 6.0 % ±7.0 of the total dietary nitrate derived from animal sources, 84.7%±12.1 from vegetables, and the remaining proportion from other food products (e.g. grains). Ingested nitrate from animal sources (mean ±SD: 5.5±2.9 mg/day) was higher among pre vs. postmenopausal women (5.9 ±2.7 vs. 5.2 ±3.0 mg/day *p value* <0.0001 for T test), but ingested nitrate from vegetable sources (mean ±SD: 110±79.6 mg/day), was lower among pre vs. postmenopausal women (96.5 ±60.4 vs. 115.0 ±84.5 mg/day *p value* <0.0001 for T test) (results not shown).

Table 2 shows the association between waterborne ingested nitrate and BC. Among postmenopausal women, fully adjusted OR (95%CI) was 1.29 (0.92, 1.81) for >8.8 mg/day compared to the lowest intake levels (<2.3 mg/day). After excluding postmenopausal women with missing or unreliable interview quality (N=118), OR (95%CI) was 1.32 (0.93, 1.86) for >8.8 vs. <2.3 mg/day. Among premenopausal women, fully adjusted OR (95%CI) was 1.14 (0.67, 1.94) for >6.3 mg/day compared to the lowest intake levels (<1.8 mg/day), and results were similar after excluding premenopausal women with unreliable interviews (N=10). Results were also similar when waterborne exposure from age 18 to 2 years before study interview was defined assuming bottled water use after 2000, for exposures from 15 to 2 years before study interview, and from age 18-30 years (see Supplemental Material, Table S2). . Exposure-response curves among study areas did not show associations except at the highest levels where estimates were extremely imprecise (see Supplemental Material. Figure S1).

Table 3 shows the associations between waterborne ingested nitrate and BC for post-menopausal women, across categories of relevant covariables. BC was inversely associated with high vitamin C+E intake (>181 mg/day) versus low vitamin C+E intake among women with low waterborne nitrate intake (<2.6 mg/day) (OR= 0.60; 95%CI: 0.39, 0.92), and the overall interaction *p value* was 0.08. However, there was no evidence that vitamin C+E intake modified the odds of BC among those in the second or third tertile of waterborne nitrate. This inverse association was not observed when vitamin C and E were analyzed separately. BC was more common among women with the highest waterborne nitrate (>6 mg/day) and highest red meat intake (>20 g/day) than among women with low waterborne nitrate (<2.6 mg/day) and low red meat intake (OR= 1.64; 95% CI: 1.08, 2.49). BC was not increased in women with high waterborne nitrate and low red meat intake (OR= 1.08; 95% CI: 0.72, 1.47), but the overall interaction between nitrate and red meat intake was not significant (LRT *p*-value= 0.17). Results for processed meat followed a similar pattern. BC was also more common among women with the highest waterborne nitrate intake and smoking history (OR= 1.48; 95% CI: 0.99, 2.21) than among women with low waterborne nitrate without smoking history, but the overall interaction was not significant (LRT *p*-value= 0.12)

Stratified analyses among premenopausal women resulted in less precise estimates of associations, due to smaller numbers of observations. Most of the ORs observed across strata were close to 1, and overall interactions were not significant (LRT p-values >0.10) (see Supplemental Material, Table S3).

Among all BC cases, 951 (76.4 %) were ductal (ICD-10 C50), 162 (13.0 %) were other malignant and *in situ* cancers (ICD-10: D05.1, D05.7), and 132 (10.6 %) were undefined. Regarding ER status, 990 (79.5 %) were positive, 218 (17.5 %) were negative and 37 (2.9 %) had missing ER status. Stratified analyses among pre and postmenopausal women combined, showed similar ORs for ductal and others/undefined tumors, as well as for positive or negative ER cancers (see Supplemental Material, Table S4).

Overall, BC was not associated with dietary nitrate from animal or vegetable sources (Table 4). The ORs reported were similar after adjusting for endogenous nitrosation factors (intake of vitamin C, vitamin E, red and processed meat)and other covariables listed in Table 1 (data not shown), or after excluding women with lower interview quality (N=128 among pre and postmenopausal women). Similar results were observed in analyses for pre and postmenopausal women separately (see Supplemental Material, Table S5).

DISCUSSION

Average waterborne ingested nitrate levels from age 18 to 2 years before the interview was 6.2 ±6.2 mg/day among controls, and 6.6 ±7.4 among cases. These levels were not associated with BC, overall. However BC in postmenopausal women was significantly increased ($p < 0.05$) in women with waterborne nitrate in the highest tertile and high red meat intake compared with waterborne nitrate in the lowest tertile and low red meat intake. Dietary ingested nitrate (mean ±SD: 125.7 ±80.3 mg/day in controls, and 123.2 ±82.3 mg/day in cases) was not associated with BC among pre or postmenopausal women, regardless of the vegetable or animal source.

To our knowledge, this is the first case-control study on ingested nitrate and BC in a European population. Most previous studies of waterborne nitrate exposure and BC have reported null associations (Brody et al. 2006; Weyer et al. 2001). A recent cohort study conducted in postmenopausal women from Iowa (N=2,875 BC cases in total), suggested an association between BC and waterborne nitrate intake in interaction with folate intake (Inoue-Choi et al. 2012). Individual data on daily water intake was not available in that study, but estimated waterborne nitrate intake levels were higher than levels in our study (median: 20 mg/day vs. 3.8mg/day), as well as the folate intake (median: 350 µg/day vs. 300 µg/day). We did not confirm an interaction with folate, probably due to the differences in nitrate and folate intake levels, and other differences, including cancer subtypes evaluated.

Stratified analyses by endogenous nitrosation factors (intake of vitamin C, vitamin E, red and processed meat), and other variables (listed in Table 1), did not show significant differences across categories, the CIs were overlapped and included the null value. BC was more frequent among postmenopausal women with highest waterborne nitrate and red meat intake than among women with low waterborne nitrate intake and low red meat intake, and the overall interaction *p*-value was >0.10. However, this joint effect is plausible, since red meat contains amines, amides, and heme iron that may increase endogenous formation of NOCs (Bingham et al. 2002). The combined intake of vitamins C and E seems to exert a protective effect, limited to postmenopausal women in the lowest tertile of waterborne nitrate intake. These findings require confirmation in future studies, since multiple stratifications were conducted and chance may not be ruled out.

The associations between waterborne ingested nitrate and BC were slightly higher in postmenopausal than in premenopausal women. However, insufficient statistical power, due to small sample size, may partly explain the null results among premenopausal women. We did not find an interaction between menopausal status and nitrate intake (*p value*=0.63) (data not shown), but we evaluated these groups separately, since differences by menopausal status have been observed with other risk factors, such as body mass index (Cheraghi et al. 2012). These differences may be attributed to endogenous hormonal production and other not well established factors. BC is a heterogeneous disease with potential different etiologies in pre and postmenopausal women; therefore the evaluation of risk factors among these subgroups may have relevant public health implications.

The evaluation of BC´s association with nitrate and other environmental pollutants in different exposure periods is required, since there is evidence suggesting that early exposure (e.g. before the first full-term pregnancy) might be the most relevant for inducing breast carcinogenesis (Brody et al. 2007). Although we evaluated three different exposure periods, we did not observe differences on the associations, probably due to high correlations between exposure levels at different periods. In addition, we did not evaluate early life exposure due to lack of data. This evaluation is warranted in future studies, particularly in settings with more available historical nitrate measurements in drinking water.

Dietary ingested nitrate levels in this study were lower than levels observed in previous studies on this topic (Inoue-Choi et al. 2012), and may partly explain the lack of statistically significant associations. Our results suggested an inverse association between

BC and ingested nitrate from vegetable sources. Vegetables contain endogenous nitrosation inhibitors (e.g. vitamins C and E), which may explain these results. Previous studies (Hord et al. 2009), suggested beneficial health effects of nitrate from vegetables sources, which might also explain these results. Further research is needed to confirm these effects and to understand the underlying mechanisms.

Potential exposure misclassification is a concern in our study since most of the long-term nitrate levels in drinking water were imputed, particularly before 1980, and water intake outside home was not accounted. However, the reported amount of water consumed at work (mean ±SD: 0.2±0.3 L/day) and other places (0.01±0.05 L/day) was smaller than at home (1.2±0.7 L/day), and minor bias is expected. We conducted sensitivity analyses excluding women with lowest interview quality, and slightly higher ORs were found, particularly among postmenopausal women. Changes on water type consumed, particularly in recent residences, may lead to exposure misclassification. To address this, we calculated an alternative variable of waterborne ingested nitrate, as explained in the Methods section. In the analysis of this alternative variable, few women (N=4 postmenopausal and N=6 premenopausal) changed exposure categories, so the associations observed (see Supplemental Material, Table S2) were similar to the main results. Potential confounding by other environmental contaminants with estrogenic action and correlated to nitrate in drinking water may occur, although available data on selected pesticides (i.e. simazine, atrazine, terbutilazine and others), showed levels below or around the quantification limit. Waterborne nitrite exposure was not evaluated either, since measurements available showed unquantifiable or extremely low levels.

Dietary nitrate estimations may also be prone to error, since nitrate content in some food items was not available, and other relevant data including vegetables´ storage and processing (i.e. washing, peeling and cooking), was not collected. Dietary nitrite intake was not evaluated, but this would not be a major limitation, since the main exposure route is through endogenous nitrate´s reduction (IARC 2010). Finally, as dietary information was collected with a FFQ, ingested nitrate misclassification due to recall bias may be a concern.

The matched case-control design by area of residence may lead to overmatching in environmental studies, and this may have occurred in this study. However, overmatching would not affect the validity of results (Agudo and González 1999). Controls had higher education level than general population, which may hamper the external validity of results. The heterogeneity of effects between some of the study areas may also be a limitation for the combined analyses.

A main strength of this study was the availability of detailed individual information on residential history, water consumption habits, and relevant covariables. Despite the limitations, the environmental data collected enabled us to evaluate BC associations for a long-term exposure window (from age 18 to 2 years before study interview), in recent years, and in early adulthood. The information provided by the FFQ allowed us to evaluate nitrate ingestion from different dietary sources and to evaluate several potential confounders and effect modifiers that were not previously evaluated, including endogenous nitrosation modulators.

In conclusion, waterborne nitrate ingestion at the exposure levels observed was not associated with BC overall. However, BC was more common among postmenopausal

women with the highest waterborne nitrate and red meat intake than among women with low waterborne nitrate and low red meat intake. Dietary nitrate was not associated with BC, regardless of the exposure source or the menopausal status.

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Table 1. Characteristics of the study population (1245 cases [a] and 1520 controls

Characteristic		Cases	Controls	*p* value*
		n (%)	n (%)	
Study area				
	Asturias	62 (5.0)	107 (7.0)	
	Barcelona	256 (20.6)	342 (22.5)	
	Cantabria	103 (8.3)	134 (8.8)	
	Gipuzkoa	171 (13.7)	246 (16.2)	
	León	155 (12.4)	168 (11.0)	
	Madrid	274 (22.0)	311 (20.5)	
	Navarra	171 (13.7)	158 (10.4)	
	Valencia	53 (4.3)	54 (3.6)	
Age				
	Mean (SD)	56.7 (12.3)	59.8 (12.9)	
	Range	23- 85	24-85	
	≤50	430 (34.5)	417 (27.4)	
	51-60	352 (28.3)	354 (23.3)	
	61-70	284 (22.8)	379 (24.9)	
	>70	179 (14.4)	370 (24.3)	
Education				
	<Primary school	184 (14.8)	266 (17.5)	
	Primary school	416 (33.4)	483 (31.8)	
	Secondary school	410 (32.9)	461 (30.3)	
	University	235 (18.9)	310 (20.4)	
Body mass index				
	<18.5	22 (1.8)	32 (2.1)	
	18.5-24.9	555 (44.6)	717 (47.2)	
	25-29.9	442 (35.5)	498 (32.8)	
	≥30	226 (18.2)	273 (18.0)	0.40
Family history of BC [a]				
	No	816 (67.5)	1,189 (81.4)	
	Yes	393 (32.5)	271 (18.6)	<0.001
	Missing	36	60	
Menopausal status				
	Premenopausal	332 (26.7)	347 (22.8)	
	Postmenopausal	913 (73.3)	1,173 (77.2)	0.02
Age at menopause [b,]				
	Mean (SD)	48.9 (5.2)	48.6 (5.2)	
	Range	28-59	22-62	
	≤50 years	460 (57.9)	634 (64.4)	
	>50 years	335 (42.1)	351 (35.6)	0.01
	Missing	450	535	

Age at menarche			
Mean (SD)	12.8 (1.6)	12.9 (1.6)	
Range	8-20	7-20	
≤12 years	535 (43.5)	608 (42.0)	
13-14 years	562 (45.7)	661 (45.7)	
≥15 years	133 (10.8)	178 (12.3)	0.45
Missing	15	73	
Age at first birth [c]			
Mean (SD)	27 (4.9)	26.8 (4.6)	
Range	16-42	15-43	
≤30 years	763 (77.7)	1,005 (80.7)	
>30 years	219 (22.3)	241 (19.3)	0.09
Missing	263	274	
Nulliparity			
No	989 (79.6)	1251 (82.6)	
Yes	253 (20.4)	264 (17.4)	0.05
Missing	3	5	
Oral contraceptives use			
Never	663 (53.4)	787 (51.8)	
Ever	579 (46.6)	732 (48.2)	0.41
Missing	3	1	
Smoking			
No	670 (54.1)	905 (59.6	
Yes	569 (45.9)	613 (40.4)	0.003
Missing	6	2	
Alcohol intake [d]			
No	384 (35.4)	516 (38.5)	
Yes	700 (64.6)	824 (61.5)	0.20
Missing	161	180	
Energy intake			
≤1479 kcal/day	288 (26.6)	447 (33.4)	
>1479-1894 kcal/day	362 (33.4)	447 (33.3)	
>1894 kcal/day	434 (40.0)	446 (33.3)	<0.001
Missing	161	180	
Vitamin C intake			
≤129 mg/day	396 (36.5)	447 (33.4)	
>129-203 mg/day	317 (29.2)	447 (33.3)	
>203 mg/day	371 (34.2)	446 (33.3)	0.08
Missing	161	180	
Vitamin E intake			
≤8.6 mg/day	356 (32.8)	447 (33.4)	
>8.6-12.2 mg/day	354 (32.7)	447 (33.3)	
>12.2 mg/day	374 (34.5)	446 (33.3)	0.82

Missing	161	180	
Folate intake			
≤252 µg/L	335 (30.9)	447 (33.4)	
>252-340 µg/L	370 (34.1)	447 (33.3)	
>340 µg/L	379 (35.0)	446 (33.3)	0.42
Missing	161	180	
Vegetables intake [e]			
≤422 g/day	393 (36.3)	447 (33.4)	
>422-642 g/day	340 (31.4)	447 (33.3)	
>642 g/day	351 (32.4)	446 (33.3)	0.31
Missing	161	180	
Red meat intake			
≤16 g/day	311 (28.7)	447 (33.4)	
>16-29 g/day	346 (31.9)	447 (33.3)	
>29 g/day	427 (39.4)	446 (33.3)	0.01
Missing	161	180	
Processed meat intake			
≤5.2 g/day	293 (27.0)	447 (33.4)	
>5.2-13.4 g/day	360 (33.2)	447 (33.3)	
>13.4 g/day	431 (39.8)	446 (33.3)	0.001
Missing	161	180	
Interview quality			
Unsatisfactory	3 (0.2)	1(0.1)	
Questionable	92 (7.5)	87 (6.1)	
Reliable	562 (46.0)	736 (51.8)	
High quality	564 (46.2)	596 (42.0)	0.02
Missing	24	100	
Histological type			
Ductal	951 (76.4)		
Others	162 (13.0)		
Undefined	132 (10.6)		
Estrogen receptors			
Positive	990 (79.5)		
Negative	218 (17.5)		
Undefined	37 (2.9)		

[a] Breast cancer cases. [b] Distribution only among postmenopausal women. [c] Distribution among non-nulliparous women. [d] Alcohol intake from age 30 to 40 years. [e] Vegetables intake include vegetables and fruits. **p*-value for Chi square test.

Table 2. Waterborne ingested nitrate from age 18 years to 2 years before study interview and breast cancer associations by menopausal status. Odds ratios (OR) and 95% confidence intervals (CI)

Menopausal status	**Cases**	**Controls**	**OR [a] (95%CI)**	**OR [b] (95%CI)**	**Cases [c]**	**Controls [c]**	**OR [b] (95%CI)**
Postmenopausal	913	1173			888	1080	
<2.3 mg/day	227	294	Ref.	Ref.	222	289	Ref.
≥2.3-4.0 mg/day	232	293	1.06 (0.82, 1.36)	1.09 (0.84, 1.41)	229	285	1.11 (0.86, 1.45)
>4.0-8.8 mg/day	222	293	1.08 (0.82, 1.41)	1.07 (0.81, 1.42)	213	263	1.10 (0.83, 1.46)
>8.8 mg/day	232	293	1.28 (0.92, 1.77)	1.29 (0.92, 1.81)	224	243	1.32 (0.93, 1.86)
p for trend			*0.19*	*0.20*			*0.16*
	Cases	**Controls**	**OR [a] (95%CI)**	**OR [d] (95%CI)**	**Cases [c]**	**Controls [c]**	**OR [d] (95%CI)**
Premenopausal	332	347			330	339	
<1.8 mg/day	72	87	Ref.	Ref.	72	86	Ref.
≥1.8-3.1 mg/day	87	87	1.24 (0.79, 1.92)	1.31 (0.83, 2.06)	86	86	1.28 (0.81, 2.02)
>3.1-6.3 mg/day	85	87	1.08 (0.68, 1.72)	1.03 (0.64, 1.66)	85	85	0.99 (0.61, 1.60)
>6.3 mg/day	88	86	1.11 (0.66, 1.86)	1.14 (0.67, 1.94)	87	82	1.05 (0.61, 1.80)
p for trend			*0.78*	*0.80*			*0.97*
	Cases	**Controls**	**OR [a] (95%CI)**	**OR [b] (95%CI)**	**Cases [c]**	**Controls [c]**	**OR [b] (95%CI)**
Pre+Postmenopausal	1245	1520			1218	1419	
<2.2 mg/day	319	380	Ref.	Ref.	315	374	Ref.
≥2.2-3.8 mg/day	303	380	0.96 (0.77, 1.19)	0.98 (0.79, 1.23)	298	373	0.98 (0.78, 1.22)
>3.8-8.1 mg/day	316	380	1.04 (0.83, 1.31)	1.01 (0.80, 1.28)	308	351	1.01 (0.80, 1.28)
>8.1 mg/day	307	380	1.09 (0.83, 1.43)	1.08 (0.82, 1.43)	297	321	1.08 (0.81, 1.44)
p for trend			*0.53*	*0.64*			*0.63*

[a] Adjusted for: study area, age and education. [b] Adjusted for: study area, age, education, body mass index, family history of breast cancer, age at first birth, age at menopause, use of oral contraceptives and energy intake. [c] Women with unreliable interviews or missing data on interview quality (N=118 postmenopausal and N=10 premenopausal) were excluded. [d] Age at menopause was excluded from the adjustment for premenopausal women. Trend *p*-values derived from a likelihood ratio test that comparing a model with the categorical nitrate intake variable as an ordinal variable (0, 1, 2) with a model that excluded this variable.

Table 3. Interaction of waterborne ingested nitrate from age 18 to 2 years before study interview with relevant dietary covariables and breast cancer associations among postmenopausal women [a]

Waterborne Ingested nitrate	Cases	Controls	OR (95% CI)	Cases	Controls	OR [b] (95% CI)	Interaction *p value**
	Vitamin C		**<170 mg/day**			**≥170 mg/day**	
<2.6 mg/day	140	162	Ref.	104	161	0.73 (0.51, 1.05)	
≥2.6-6.0 mg/day	118	169	0.88 (0.62, 1.24)	153	171	1.08 (0.77, 1.52)	
>6.0 mg/day	146	185	1.19 (0.80, 1.77)	136	183	1.06 (0.72, 1.58)	0.10
	Vitamin E		**<10 mg/day**			**≥10 mg/day**	
<2.6 mg/day	131	170	Ref.	113	153	0.84 (0.58, 1.22)	
≥2.6-6.0 mg/day	132	160	1.18 (0.83, 1.67)	139	180	0.94 (0.65, 1.34)	
>6.0 mg/day	123	186	1.16 (0.77, 1.74)	159	182	1.25 (0.82, 1.88)	0.41
	Vitamin C+E		**<181 mg/day**			**≥181 mg/day**	
<2.6 mg/day	142	161	Ref.	102	162	0.60 (0.39, 0.92)	
≥2.6-6.0 mg/day	116	167	0.94 (0.62, 1.42)	155	173	1.09 (0.73, 1.63)	
>6.0 mg/day	145	185	1.17 (0.73, 1.89)	137	183	1.02 (0.64, 1.64)	0.08
	Folate		**<300 µg/day**			**≥300 µg/day**	
<2.6 mg/day	143	169	Ref.	101	154	0.74 (0.52, 1.07)	
≥2.6-6.0 mg/day	116	173	0.89 (0.63, 1.25)	155	167	1.09 (0.78, 1.54)	
>6.0 mg/day	135	174	1.19 (0.80, 1.77)	147	194	1.09 (0.73, 1.62)	0.13
	Red meat		**<20 g/day**			**≥20 g/day**	
<2.6 mg/day	104	151	Ref.	140	172	1.03 (0.72, 1.47)	
≥2.6-6.0 mg/day	119	160	1.13 (0.78, 1.63)	152	180	1.17 (0.82, 1.67)	
>6.0 mg/day	124	205	1.08 (0.71, 1.62)	158	163	1.64 (1.08, 2.49)	0.17
	Processed meat		**<7.2 g/day**			**≥7.2 g/day**	
<2.6 mg/day	118	180		126	143	1.09 (0.76, 1.56)	
≥2.6-6.0 mg/day	116	165	1.12 (0.79, 1.60)	153	175	1.24 (0.88, 1.75)	
>6.0 mg/day	100	171	1.14 (0.76, 1.70)	182	197	1.62 (1.08, 2.44)	0.46
	Smoking		**No**			**Yes**	
<2.6 mg/day	161	226	Ref.	118	141	Ref.	
≥2.6-6.0 mg/day	188	247	1.12 (0.84, 1.51)	116	147	0.93 (0.66, 1.32)	
>6.0 mg/day	197	296	1.13 (0.80, 1.60)	128	115	1.48 (0.99, 2.21)	0.12

[a] Only women with complete information on dietary covariables (N=1828) or smoking (N=2080) were analyzed. [b] Adjusted for: study area, age, education, body mass index, family history of breast cancer, age at menopause, age at first birth, oral contraceptives use, and energy intake. **p*-value for overall interaction calculated by comparing the multivariate models with and without the interaction term using a likelihood ratio test

Table 4. Odds ratio (OR) of breast cancer associated with dietary ingested nitrate (mg/day) from different sources (N=2424) [a]

Ingested nitrate from:	Cases	Controls	OR [b] (95%CI)	OR [c] (95%CI)
Animal sources				
<4.0 mg/day	307	447	Ref.	Ref.
4.0-<6.0mg/day	364	447	1.12 (0.92, 1.38)	1.02 (0.82, 1.26)
≥6.0mg/day	413	446	1.19 (0.97, 1.47)	1.04 (0.83, 1.31)
p for trend			*0.09*	*0.72*
Vegetables sources				
<76mg/day	385	447	Ref.	Ref.
76-122 mg/day	355	447	0.92 (0.75, 1.12)	0.90 (0.74, 1.11)
>122mg/day	344	446	0.90 (0.74, 1.11)	0.86 (0.69, 1.06)
p for trend			*0.33*	*0.15*
Total diet				
<90 mg/day	387	447	Ref.	Ref.
90-138 mg/day	349	447	0.90 (0.74, 1.10)	0.86 (0.70, 1.06)
>138 mg/day	348	446	0.90 (0.73, 1.10)	0.83 (0.67, 1.03)
p for trend			*0.30*	*0.09*
Total diet+waterborne				
<96 mg/day	386	447	Ref.	Ref.
96-144 mg/day	341	447	0.89 (0.73, 1.09)	0.84 (0.69, 1.04)
>144 mg/day	357	446	0.94 (0.76, 1.15)	0.87 (0.70, 1.08)
p for trend			*0.51*	*0.19*

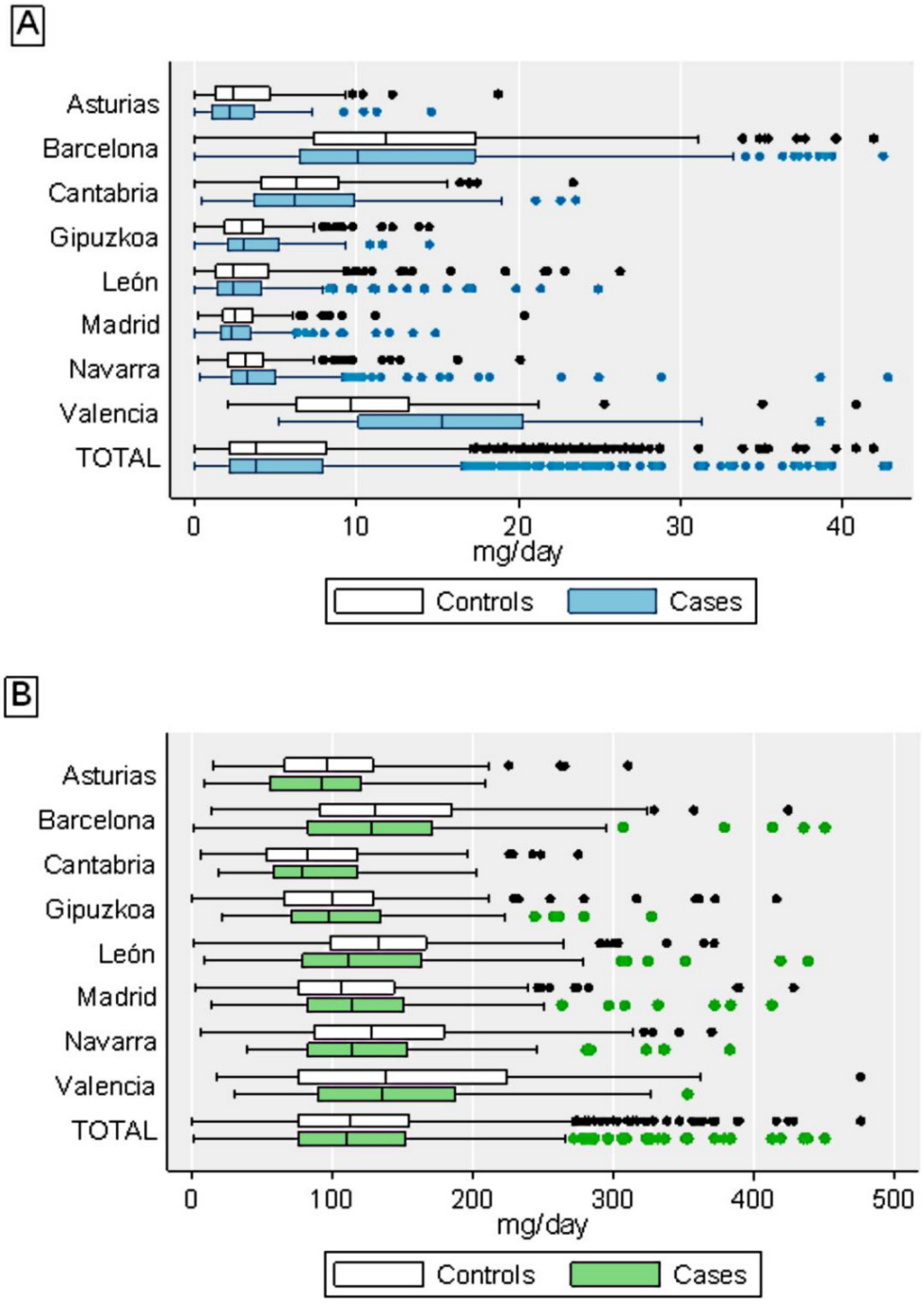
[a] Only women with available data from the food frequency questionnaire was analyzed.

[b] Adjusted for study area, age and education. [c] Adjusted for study area, age, education, body mass index, family history of breast cancer, use of oral contraceptives, and energy intake. Trend *p*-values derived from a likelihood ratio test that comparing a model with the categorical nitrate intake variable as an ordinal variable (0, 1, 2) with a model that excluded this variable.

Figure legend

Figure 1. Ingested nitrate levels (mg/day) through drinking water from age 18 to 2 years before the interview (A) and diet (B) among study areas. Women with waterborne ingested levels >44 mg/day (N=6) or with dietary ingested levels >476 mg/day (N=7) were excluded from the graphics. Boxes extend from the 25^{th} to the 75^{th} percentile. Horizontal bars represent the median, whiskers indicate the 10^{th} and 90^{th}, and outliers are represented as points.

Figure 1.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