Nitrate exposure and cancer risk

Evidence from European case-control studies

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A mi hermano pequeño Carlos
(1987-2012)

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"…Gracias a la vida que me ha dado tanto
Me dio el corazón que agita su marco
Cuando miro el fruto del cerebro humano,
Cuando miro al bueno tan lejos del malo,
Cuando miro al fondo de tus ojos claros.

Gracias a la vida que me ha dado tanto
Me ha dado la risa y me ha dado el llanto,
Así yo distingo dicha de quebranto
Los dos materiales que forman mi canto
Y el canto de ustedes que es el mismo canto
Y el canto de todos que es mi propio canto"

(Violeta Parra 1966)

Abstract

Background and aims: Nitrate is widespread in the environment and ingestion of drinking water and diet are the most relevant sources of human exposure. Ingested nitrate leads to the endogenous formation of N-nitroso compounds (NOCs), which are animal carcinogens with limited human evidence. The main objective of this thesis was to evaluate the association of prevalent tumors with nitrate exposure through drinking water and diet, in the context of three large European case-control studies: the Spanish Bladder Cancer study, the Spanish Multi-case Control study on Cancer (MCC-Spain) and the HIWATE project in Italy.

Methods: Incident cases of bladder, breast and colorectal cancer and matched controls were interviewed on lifetime residential history, water consumption habits, and dietary information. Current nitrate levels were measured in samples of municipal water (n=227) from 11 Spanish regions, and in samples of non-municipal water (bottled water n=9, and well water n=28). Long-term nitrate levels (1940-2010) were derived from monitoring data provided by local authorities and water companies in municipalities of residence. Residential information was linked to long-term nitrate levels by municipality and year. Then, individual exposure since age 18 onwards was estimated, according to individual water consumption habits (type of water consumed in the residence and amount of water daily intake). Ingested nitrate through diet (animal and vegetable sources) was estimated based on data from food frequency questionnaires and published food composition databases. Associations of each tumor with nitrate exposures were estimated by logistic regression analyses. Odds ratios (OR) and confidence intervals (95%CI) were adjusted for specific risk factors. Several potential confounders were tested including endogenous nitrosation factors. Effect modification by relevant variables for each tumor was also evaluated. Generalized additive models (GAMs) and other analyses were applied to evaluate the exposure-response relationship between nitrate exposure and cancer risk. The population analyzed comprised 531 bladder cancer cases and 556 controls, 1245 breast cancer cases and 1520 controls, and 1869 colorectal cancer cases and 3530 controls.

Results: Nitrate levels in municipal drinking water from 67 Spanish municipalities resulted below the maximum regulatory limit (50mg/L of NO_3^-) (median: 4.2 mg/L, range: <1.0-29.0 mg/L). Low nitrate levels were found in samples of bottled water brands.

Long-term nitrate exposure levels through drinking water differed between study areas (12 regions in Spain and 2 in Italy). Overall, residential nitrate levels (mean±SD) ranged from 1.6±0.9 to 30.0±4.4 mg/L and waterborne ingested nitrate ranged from 2.9± 1.9 to 19.7±22.6 mg/day.

Higher risk of bladder cancer was observed only for subjects with residential nitrate levels >9.5 mg/L for >20 years. Non-statistically significant inverse association was found for waterborne ingested nitrate, but residual confounding by the protective effect of water intake on bladder cancer may not be ruled out. Results were not modified by endogenous nitrosation factors (e.g. vitamin C intake) or trihalomethane levels in drinking water. Higher risk of breast cancer was found among postmenopausal women with both highest waterborne nitrate and highest red or processed meat intake. Non-statistically significant increased risk of colorectal cancer was observed with long-term exposure to nitrate in drinking water, but heterogeneous exposure-response relationships were observed among study areas. Higher risk was observed among men compared to women (p value for interaction <0.009), and among a subgroup of men with simultaneous high waterborne nitrate and meat intake.

Dietary ingested nitrate (mean±SD) ranged from 78.1±48.6 to 154±68.4 mg/day, among regions. Most of the ingested nitrate was provided by vegetable sources. These levels were not associated with breast cancer risk, regardless the menopausal status or the ingestion source (vegetable or animal). Only the nitrate ingested from animal sources was associated with rectal cancer risk.

Conclusion: Long-term nitrate exposure, at levels below the current guidelines for drinking water, increased cancer risk only in subgroups of the population. Overall, heterogeneous effects were observed with ingested nitrate from different sources (water, animal and vegetables). These results require confirmation in settings with wider range of exposure and improved methods for exposure assessment.

Resumen

Introducción y objetivos: El nitrato está ampliamente diseminado en el ambiente. La ingestión a través del agua de consumo y la dieta es la vía más importante de exposición humana. Una vez ingerido, el nitrato da lugar a la formación endógena de productos N-nitroso (NOCs) que son carcinógenos en animales, con poca evidencia en humanos. El objetivo principal de esta tesis es evaluar la asociación entre tumores prevalentes y la exposición a nitrato a través del agua de consumo y la dieta, en el marco de tres grandes estudios Europeos de caso-control: el estudio español de Cáncer de Vejiga (*Spanish Bladder Cancer Study*), el estudio español Multicaso-control de cáncer (*MCC-Spain*) y el proyecto HIWATE en Italia.

Métodos: Casos incidentes de cáncer de vejiga, mama y colorrectal y sus respectivos controles fueron entrevistados sobre su historial residencial completo, hábitos de consumo de agua y de dieta. Los niveles actuales de nitrato fueron analizados en muestras de agua municipal (n=227) de 11 regiones españolas y en muestras de agua no municipal (embotellada n=9 y de fuentes n=28). Niveles históricos de nitrato (1940-2010) fueron estimados a partir de datos de monitoreo proporcionados por las autoridades locales y compañías de agua, en los municipios de residencia. El historial residencial y los niveles históricos de nitrato fueron unidos por municipio y por año. Se estimó la exposición individual desde los 18 años, de acuerdo a los hábitos de consumo de agua (tipo de agua consumido en las residencias y cantidad de agua ingerida por día). La ingestión de nitrato a través de la dieta se estimó a partir de los datos de un cuestionario de frecuencia alimentaria y datos publicados sobre composición de alimentos. Las asociaciones entre cada tumor y las variables de exposición a nitrato se estimaron por análisis de regresión logística. Odds ratios (OR) e intervalos de confianza (95%CI) fueron ajustados por factores de riesgo específicos. Varios potenciales confusores, incluyendo factores de nitrosación endógena, fueron evaluados. Varios potenciales modificadores de efecto fueron también evaluados para cada tumor. *Generalized Additive Models* (GAMs) y otros análisis se aplicaron para evaluar la relación exposición-respuesta entre niveles de nitrato y el riesgo de cáncer. La población analizada incluyó 531 casos de

cáncer de vejiga y 556 controles, 1245 casos de cáncer de mama y 1520 controles y 1869 casos de cáncer colorrectal y 3530 controles.

Resultados: Los niveles de nitrato en muestras de agua de consumo municipal resultaron menores al nivel regulatorio vigente en Europa (50 mg/L de NO_3^-) (mediana: 4.2 mg/L, rango: <1.0-29.0 mg/L). Así mismo, se detectaron niveles bajos de nitrato en muestras de agua embotellada españolas.

Los niveles de exposición a nitrato a largo plazo en agua de consumo fueron diferentes entre las áreas de estudio (12 regiones en España y 2 en Italia). Los promedios de niveles residenciales de nitrato (media± DE) estuvieron entre 1.6±0.9 y 30.0±4.4 mg/L y los de niveles ingeridos en agua de consumo entre 2.9± 1.9 y 19.7±22.6 mg/día. Se observó un mayor riesgo de cáncer de vejiga sólo en personas expuestas a niveles residenciales de nitrato >9.5 mg/L por más de 20 años. Se observó también una asociación inversa con niveles de nitrato ingerido en agua de consumo, pero no se puede descartar una posible confusión residual por el efecto protector de la ingesta de agua. Estos resultados no se modificaron al tomar en cuenta factores de nitrosación endógena (ej. ingesta de carne roja) o niveles de trihalometanos en agua municipal. Se detectó un incremento de riesgo de cáncer de mama en mujeres postmenopáusicas con niveles más altos de ingesta de nitrato en agua de consumo y de carne roja o procesada. Se observó riesgo elevado, pero no estadísticamente significativo, de cáncer colorrectal asociado a la exposición a nitrato en agua de consumo, aunque la relación exposición-respuesta fue heterogénea entre las áreas de estudio. El riesgo observado fue más elevado en hombres que en mujeres (*p valor* de interacción <0.009) y en hombres con mayor ingesta simultánea de nitrato en agua de consumo y de carne.

Los niveles de nitrato ingerido a través de la dieta (media± DE) resultaron en el rango de 78.1±48.6 a 154±68.4 mg/día entre las regiones de estudio. La mayor parte del nitrato ingerido fue provisto por fuentes vegetales. Estos niveles no se asociaron con riesgo de cáncer de mama, independientemente del estado menopáusico o la fuente de consumo animal o vegetal. Sólo el nitrato ingerido de fuentes animales estuvo asociado a un incremento de riesgo de cáncer rectal.

Conclusión: La exposición a largo plazo a nitrato, por debajo de los niveles regulados vigentes en agua de consumo, incrementó el riesgo de cáncer sólo en subgrupos de la población estudiada. Se observaron efectos heterogéneos entre las diferentes fuentes de ingestión de nitrato (agua y dieta: fuentes animal y vegetal). Estos resultados requieren confirmación en escenarios con mayores rangos de exposición y métodos optimizados para la evaluación de la exposición.

Resum

Introducció i objectius: El nitrat està amplament estès en l'ambient. La ingestió a través de l'aigua de consum i la dieta és la via més important d'exposició humana. Una vegada ingerit, el nitrat dóna lloc a la formació endògena de productes N-nitrós (NOCs) que són substàncies cancerígenes en animals, amb limitada evidència en humans. L'objectiu principal d'aquesta tesi és avaluar l'associació entre tumors prevalents i l'exposició del nitrat a través de l'aigua de consum i la dieta, en el marc de tres grans estudis Europeus de cas-control: l'estudi espanyol de Càncer de Bufeta *(Spanish Bladder Cancer Study)*, l'estudi Multicas-control de càncer a Espanya *(MCC-Spain)* i el projecte HIWATE a Itàlia.

Mètodes: Casos incidents de càncer de bufeta, mama i colorrectal i els seus controls respectius van ser entrevistats sobre el seu historial residencial complet, hàbits de consum d'aigua i de dieta. Els nivells actuals de nitrat van ser analitzats en mostres d'aigua municipal (n=227) d'onze regions espanyoles i en mostes d'aigua no municipal (embotellada n=9 i de fonts n=28). Els nivells històrics de nitrat (1940-2010) van ser estimats a partir de dades de seguiment proveïts per autoritats locals i companyies d'aigua en els municipis de residència. El historial residencial i els nivells històrics de nitrat van ser aparellats per municipi i per any. Després s'ha estimat l'exposició individual des dels 18 anys en endavant d'acord amb els hàbits de consum d'aigua (tipus d'aigua consumida en les residències i quantitat d'aigua ingerida per dia). La ingesta de nitrat a través de la dieta va ser estimada a partir de dades d'un qüestionari de freqüència alimentaria i dades publicades sobre composició d'aliments. Les associacions entre cada tumor i les variables d'exposició a nitrat van ser estimades per anàlisis de regressió logística. *Odds ratios* (OR) i intervals de confiança (95% CI) van ser ajustats per factors de risc específics incloent factors de nitrosació endògena. També van ser avaluats diferents potencials modificadors d'efecte per cada tumor. *Generalized Additive Models* (GAMs) i altres analitzis es van aplicar per avaluar la relació exposició-resposta entre els nivells de nitrat i el risc de càncer. La població analitzada va incloure: 531 casos de càncer de bufeta i 556 controls, 1245 casos de càncer de mama i 1520 controls i 1869 casos de càncer colorrectal i 3530 controls.

Resultats: Els nivells de nitrat en mostres d'aigua de consum municipal van resultar menors al nivell establert vigent a Europa (50 mg/L de NO_3^-) (mediana: 4.2 mg/L, rang: <1.0-29.0 mg/L). Així mateix, es van detectar nivells baixos de nitrat en mostres d'aigua embotellada espanyoles.

Els nivells d'exposició de nitrat a llarg termini en aigua de consum van diferir entre les àrees d'estudi (12 regions d'Espanya i 2 d'Itàlia). Les mitjanes de nivells residencials de nitrat (mitjana±DE) van registrar-se entre 1.6±0.9 y 30.0±4.4 mg/L, i els nivells ingerits en aigua de consum entre 2.9± 1.9 y 19.7±22.6 mg/día. S'ha observat major risc de càncer de bufeta únicament en persones exposades a nivells residencials de nitrat >9.5 mg/L durant 20 anys o més. També es va observar una associació inversa amb els nivells de nitrat ingerit en aigua de consum, però no es pot descartar una possible confusió residual per l'efecte protector de la ingesta d'aigua. Aquests resultats no es van modificar al tenir en compte factors de nitrosació endògena (ex: ingestió de carn vermella) o nivells de trihalometans en aigua municipal. Es va detectar un increment de risc de càncer de mama en dones post-menopàusiques amb nivells més alts d'ingestió de nitrat en aigua de consum i de carn vermella o processada. Es va observar un risc elevat, però no estadísticament significatiu, de càncer colorrectal associat a l'exposició de nitrat en aigua de consum, encara que la relació exposició-resposta va ser heterogènia entre les àrees d'estudi. El risc observat va ser més elevat en homes que en dones (*p valor* d'interacció <0.009) i en homes amb major ingestió simultani de nitrat en aigua de consum i de carn.

Els nivells de nitrat ingerit a través de la dieta (mitjana±DE) van resultar en el rang de 78.1±48.6 i 154±68.4 mg/día, entre regions. La major part del nitrat ingerit prové de fonts vegetals. Aquests nivells no es van associar amb risc de càncer de mama, independentment de l'estat menopàusic o de la font de consum animal o vegetal. Només el nitrat ingerit de fonts animals va estar associat amb un increment de risc de càncer rectal.

Conclusió: L'exposició a llarg termini de nitrat, per sota dels nivells regulats vigents per aigua de consum, va incrementar el risc de càncer en alguns subgrups de la població analitzada. Es van observar efectes heterogenis entre les diferents fonts d'ingestió de nitrat (aigua i dieta: fonts animal i vegetal). Aquests resultats requereixen confirmació en medis amb majors rangs d'exposició i mètodes optimitzats per l'avaluació de l'exposició.

Preface

Nitrate is one of the most frequent contaminants in drinking water worldwide. Nitrate is also a main food component of vegetables and processed meat products, which are frequently consumed among population of western countries. Furthermore, ingested nitrate leads to endogenous formation of N-nitroso compounds, which are carcinogens in animals, but few studies evaluating the potential carcinogenicity of nitrate are available in humans. Given the state of the evidence, nitrate was classified as a probable human carcinogen in 2006. The main issue addressed in this thesis is whether the exposure to nitrate through drinking water and diet is associated with carcinogenic effects in humans.

This thesis was conducted in the Centre for Research in Environmental Epidemiology (CREAL) between 2011 and 2015, under the supervision of Cristina M. Villanueva Belmonte PhD. The thesis consists of a compilation of four scientific papers, according to the requirements of the Doctoral Program in Biomedicine of the Pompeu Fabra University. This document also includes a general introduction, a description of the methodology, an overall discussion of the results, conclusions and an appendix section.

The core of the thesis include: a) the results of the largest tap water sampling conducted to date for nitrate determination in Spain (paper I), and b) the results of three large case-control studies evaluating the risk of relevant tumors (bladder, breast and colorectal) associated with nitrate exposure through drinking water and diet (paper II, III and IV). These studies are among the few analyses conducted on European population.

The main findings of this thesis provide evidence on potential cancer effects in humans associated with nitrate exposure in drinking water at levels below the current European regulatory limit. These results also allow the identification of subgroups of the population with higher cancer risk associated with nitrate ingestion (e.g. subjects with high meat intake). Additionally, the findings suggest heterogeneous effects of nitrate exposure by the ingestion source (drinking water or diet from animal or vegetable sources).

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1 Introduction

1.1 Nitrate in the environment

Nitrate is naturally ubiquitous in the environment, and is part of the nitrogen cycle (**Figure 1**). Nitrogen is the most abundant element in the atmosphere but is chemically inert, thus is converted into biologically active forms, such as nitrate (NO_3^-) and nitrite (NO_2^-). This conversion is essential to maintain life on earth and requires multistep enzymatic pathways and a considerable amount of energy (Gilchrist and Benjamin 2011). Nitrogen compounds from organic residues (e.g. sewage and human waste) are also converted into nitrate and nitrite in soils, through reactions mediated by bacteria (Carter et al. 1995). Afterwards, plants uptake and accumulate a part of this pool of nitrate, while another part is reduced to dinitrogen gas (N_2), and other gaseous forms by denitrification, that circulate through the atmosphere.

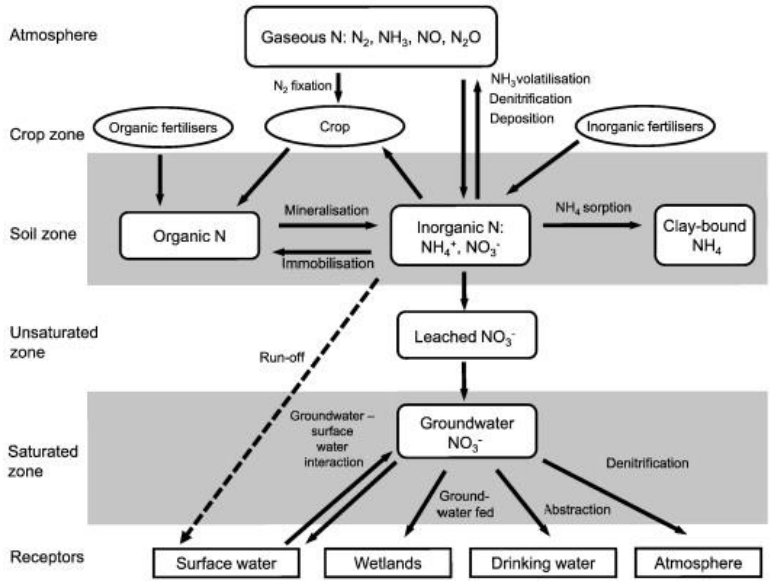


Figure 1. Nitrogen cycle in the environment highlighting soil processes. (Source: (Stuart et al. 2011).

This natural cycle has been disturbed in the last decades, resulting in nitrate accumulation in soils and contamination of water sources. Several human activities are related to this environmental disruption, the most relevant are: the increasing and excessive use of nitrogen based fertilizers for agricultural purposes (Randall and Mulla 2001), intensive sewage and accumulation of human waste in urban areas (Wakida and Lerner 2005).

1.1.1 Nitrate levels in drinking water

Highest nitrate levels are usually observed in ground water sources from agricultural areas (Burkart and Stoner 2007), whereas levels in surface sources do not exceed 10 mg/L of nitrate as ion NO_3^- (Aelion and Conte 2004). Ground water reservoirs (aquifers) located in areas with well drained soils and unconsolidated rocks are the most susceptible to nitrate contamination (Ward et al. 2005).

Nitrate levels in water sources differ widely between countries, and are increasing in some regions including developing countries (**Figure 2**). In Europe, high nitrate levels in stream and ground water sources were reported in several areas, mainly due to overuse of fertilizers in agriculture. Countries like United Kingdom, Germany and Belgium, still show high nitrate levels in surface and ground water sources (**Figure 3**).

In Spain, average nitrate levels in raw stream water from agricultural areas ranged from 1.3 to 40.3 mg/L of NO_3^-, with increasing trends during 1981-2005 (Lassaletta et al. 2009). High levels have been also described in drinking water from Valencia, an agricultural region (Vitoria Miñana et al. 1991), but the information from other regions is sparse (Caballero Mesa et al. 2003; Salgado et al. 2003). Only since the year 2003, monitoring nitrate levels in water from public distribution systems are centralized by the National Information System in Drinking Water (*Sistema Nacional de Información en Aguas de Consumo)*(SINAC 2011).

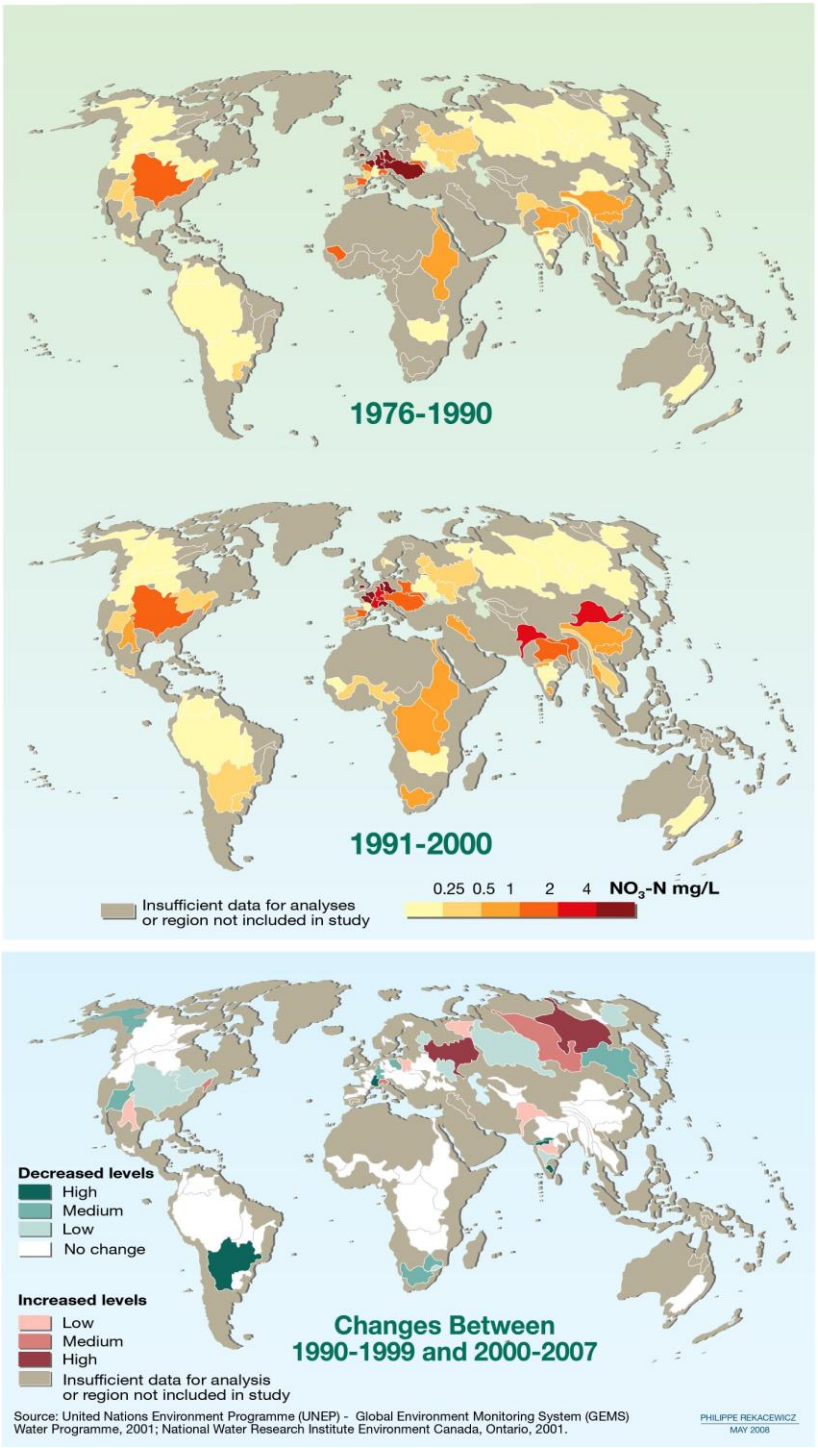


Figure 2. Nitrate levels in surface sources worldwide

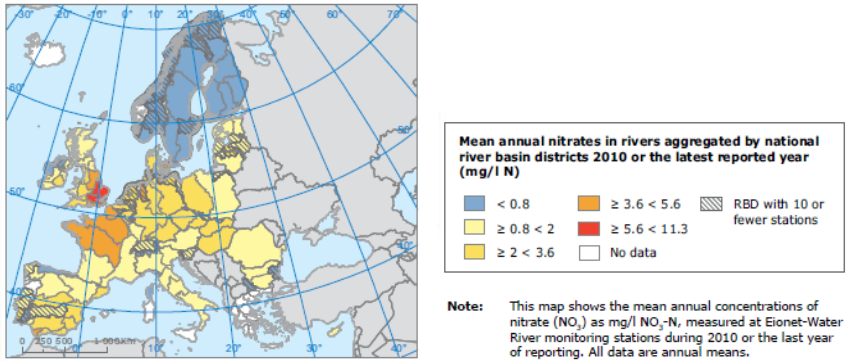


Figure 3: Nitrate levels in European surface water 2010. Source (EEA 2012)

1.1.2 Regulation of nitrate levels in drinking water

Some studies were published around the year 1950 describing adverse effects (methemoglobinemia) in infants exposed to high nitrate levels through consumption of tap water based formula (Donahoe 1949; Walton 1951) To prevent this acute effect, the World Health Organization (WHO) established a maximum contaminant limit (MCL) of **50 mg/L of nitrate as ion (NO_3^-)** in drinking water, and confirmed this regulatory limit in 2008 (WHO 2008). In the United States of America the regulation was adapted to 11.3 mg/L of nitrate as nitrogen (N-nitrate) and modified to **10 mg/L of nitrate as nitrogen (N-nitrate)** in 1991 (US-EPA 1991). The WHO guideline was also adapted to Spanish regulation, and reconfirmed in 2003 (BOE 2003). In addition to the international legislation, the Council of European Communities released the "Nitrate Directive" in 1991 (EEC 1991), aimed to reduce water pollution caused by nitrates from agricultural sources in Europe.

1.1.3 Nitrate dietary sources

Diet is the most important source of human exposure to nitrate, particularly when the levels in drinking water are below the international regulatory limits (Levallois et al. 2000). The amount of nitrate intake through food, estimated previously, ranged from 31 to 185 mg/day in Europe, and from 40 to100 mg/day in the United States. Around 80% of the intake was derived from vegetables (Hord et al. 2009). Highest nitrate contents are naturally observed in

green leafy vegetables (e.g. rocket salad or spinach), but a wide range of variation was observed according to vegetable types, regions of origin, and other several factors (e.g. exposure to fertilizers, season of harvest and storage) (EFSA 2008).

Nitrate and nitrite are also ingested through cured meat and other food products. Nitrate salts (e.g. potassium nitrate) inhibit bacterial growth in meat, contributing to their safety for human consumption. Curing has been historically used for preserving food, and currently no suitable alternative is available (Keeton 2011). The consumption of such food products is frequent among European population. Moreover, the production of cured meat products is an important economic activity and an ancient tradition in some Spanish regions and other European countries (**Figure 4**). Current international regulations allow the use of nitrite and selective use of nitrate in meat products based on product category and method of curing (EEC 1995). The WHO established an acceptable daily intake (ADI) of **3.7 mg/kg body weight/day** of nitrate as ion (0-5 mg/kg body weight/day of sodium nitrate). This ADI is equivalent to **222 mg/day for a 60 kg adult** (WHO/FAO 2002).

Figure 4. "The Pig-slaughtering day" in Basturs (Catalonia, Spain).

1.2 Nitrate in the human body

1.2.1 Endogenous nitrosation: from ingested nitrate to nitrite and N-nitroso compounds

Once ingested, nitrate is totally absorbed in the gut and follows an entero-salivary circulation. Around 65% of the nitrate absorbed is secreted in saliva (Bryan and Loscalzo 2011). Salivary nitrate is converted into nitrite through chemical reduction mediated by commensal anaerobic bacteria in the oral cavity (Lundberg and Govoni 2004). It has been observed that changes in oral bacteria, by factors like the use of antibacterial mouth washes (chlorhexidine), may influence the conversion of nitrate into nitrite (van Maanen et al. 1998). Nitrite is catabolized to nitric oxide (NO) and nitrous acid (HNO_2) in acidic conditions of the gastric lumen. In these conditions, HNO_2 may be combined to amines and amides groups, leading to the synthesis of N-nitrosamines and N-nitrosamides. An example of NOCs synthesis is shown in **Figure 5.** These nitrate derivatives are known as N-nitroso compounds (NOCs). The synthesis of NOCs in the human body is known as endogenous nitrosation (**Figure 6**), and accounts for 45-75% of the total human exposure to N-nitroso compounds (Tricker 1997).

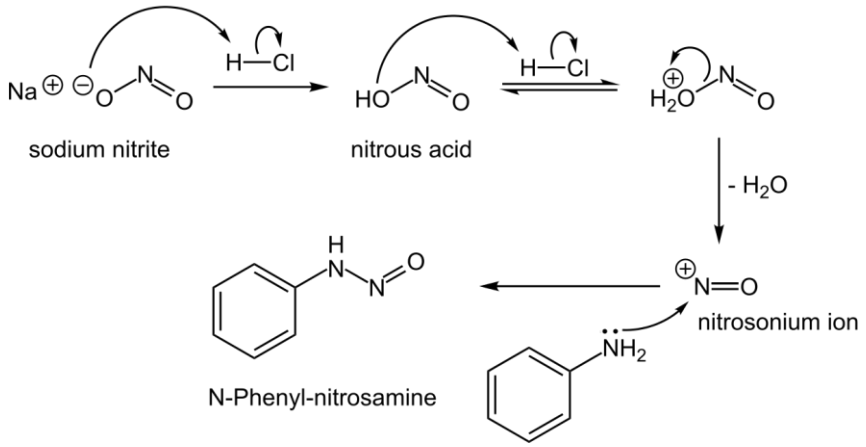


Figure 5. Nitrosamines synthesis

Endogenous nitrosation is also produced in other organs, including urinary bladder. Approximately 60% of the nitrate ingested or endogenously synthesized is excreted in urine within 48 hours (Bryan and Loscalzo 2011). Urinary nitrate is converted into nitrite in presence of nitrate-reducing microorganisms, and follows the described pathway until NOCs formation.

1.2.2 Modulators of endogenous nitrosation

Several factors interplay to modulate endogenous nitrosation. Inhibitors factors include: the intake of antioxidant vitamins (vitamin C and E), polyphenols and other micronutrients (Bartsch et al. 1988), and the use of non-steroidal anti-inflammatory drugs (NSAIDs). On the contrary, the intake of red meat or processed meat, increased acidic conditions (e.g. in subjects with gastric ulcer diagnosis), the presence of inflammatory conditions in the gastrointestinal tract (e.g. Crohn´s disease) (Dietrich et al. 2005), and the intake of Heme iron (Bastide et al. 2011), were identified as endogenous nitrosation promoters.

1.2.3 Exogenous sources of NOCs

Apart from endogenous nitrosation, humans may be exposed to exogenous NOCs, mainly through diet. Cured meat is also a main dietary source of pre-formed NOCs, such as N-nitrosodimethylamine (NDMA) (Jakszyn et al. 2006). Nitrite and nitrate, added during the curing process, are converted into NOCs by bacterial and enzymatic reactions, and are ingested through cured meat. Other relevant sources of preformed NOCs are smoking cigarettes and alcoholic beverages, although nitrate contents reported in these food products may differ widely among reports (Jakszyn et al. 2004).

1.3 Carcinogenic effects mediated by NOCs

1.3.1 Evidence from mechanistic and animal studies

Carcinogenic effects of ingested nitrate depend on the stepwise conversion into nitrite and the endogenous formation of NOCs. NOCs may induce carcinogenic changes by different mechanisms including cytotoxicity, mutagenicity, genotoxicity and epigenetic changes (e.g. DNA- methylation). These effects were observed in *in vitro* and *in vivo* studies (Archer 1989). NOCs become potent electrophilic alkylating agents in the human body. This process is spontaneous for nitrosamides, but requires previous activation for nitrosamines, mediated by the human cytochrome P450-system (iso-enzymes CYP1A2, CYP2A3, CYP2E1 and CYP2D6) (Crespi et al. 1991). NOCs, as alkylating agents, react with DNA leading to the formation of DNA adducts and the induction of carcinogenesis. Mutations in genes *ras* and *p53* are also produced by alkylating agents like NOCs (Hebels et al. 2011).

The exposure to NOCs induced cancer effects in several animal species including mammals (Bogovski and Bogovski 1981). Carcinogenic changes were observed in various organs including liver, esophagus, and stomach among these species (Verna et al. 1996). Tumors in lung, colon, uterus and mammary glands were also observed in rodents (Lijinsky et al. 1992). Some NOCS, such as N-methyl-N-nitrosourea (MNU), are used to induce breast cancer in experimental studies with animals. One study with rodents showed that rats exposed to MNU at early ages showed more susceptibility to develop breast tumors (Tsubura et al. 2011). Overall, the evidence suggests that biological activity of NOCs in humans is substantially similar to that observed in experimental animals.

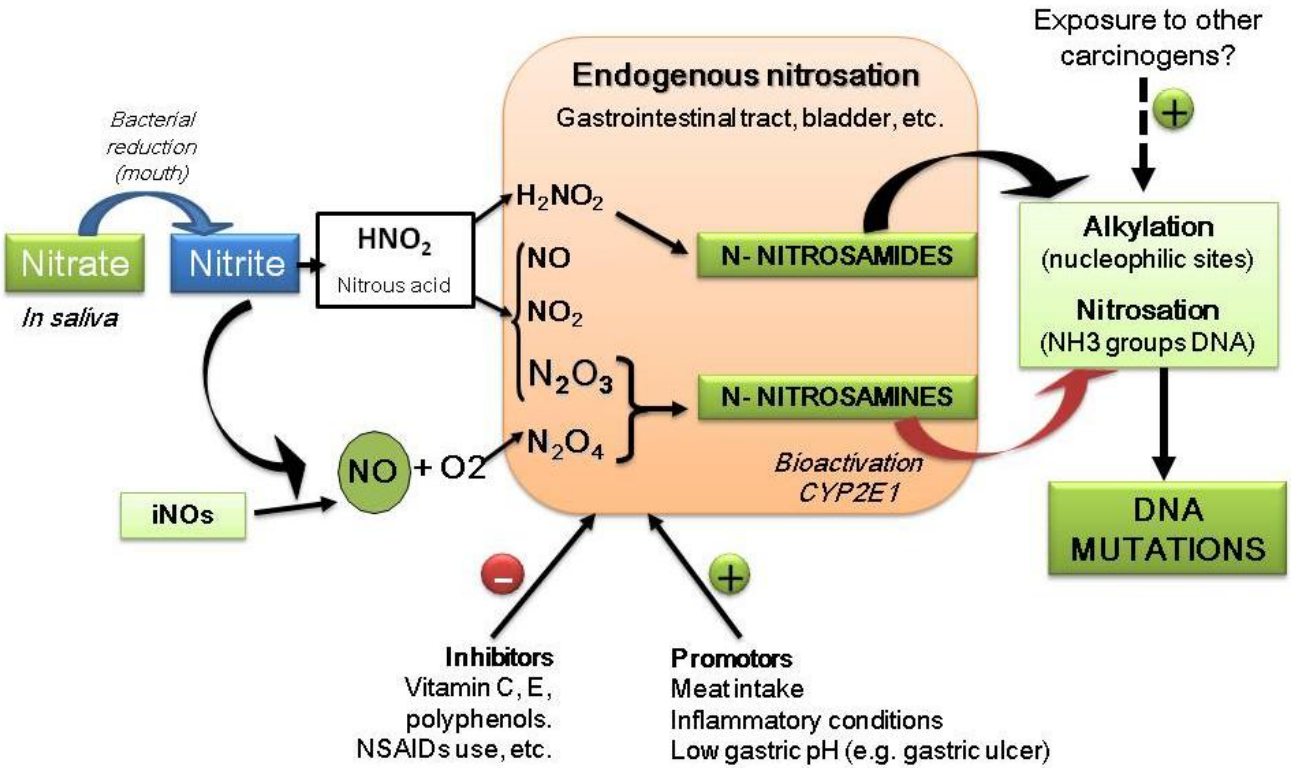


Figure 6. Endogenous nitrosation and carcinogenic mechanisms of NOCs (Schematic diagram based on Hord et al. 2009).

1.3.2 Evidence from epidemiologic studies

In contrast to the evidence from animal studies, the evidence from epidemiologic studies in human populations is inconclusive (Grosse et al. 2006). Therefore, nitrate was classified as a probable human carcinogen in conditions of endogenous nitrosation by the International Agency of Research on Cancer, and classified in the group 2A (IARC 2010). The following paragraphs summarize the epidemiologic evidence available on nitrate exposure through drinking water and diet, and the tumors evaluated in this thesis (bladder, breast and colorectal cancer). **Table 1** and **Table 2** show additional information on the evidence available.

a) Nitrate exposure and bladder cancer

An ecological study conducted in Spain suggested higher risk of bladder cancer mortality or cancer incidence associated with high nitrate levels in drinking water (>50 mg/L of NO_3^-) (Morales Suarez-Varela et al. 1993). In contrast, no association was found in an ecological study from the Slovak Republic, among villages with nitrate levels in drinking water of 20-50 mg/L vs. villages with levels of 0-10 mg/L (Gulis et al. 2002). These studies did not consider individual information such as water consumption, occupational history, or exposure to endogenous nitrosation modifiers.

A case-control study from the U.S. observed a reduced risk of bladder cancer with waterborne nitrate exposure. The OR (95%CI) for levels ≥3.09 mg/L vs. <0.6 mg/L of nitrate-N was 0.8 (0.4-0.8) among women and 0.5 (0.4-0.8) among men, regardless of vitamin C intake (Ward et al. 2003)

Regarding cohort studies: an analysis from the U.S. found a positive association with bladder cancer and nitrate-N levels in drinking water (RR= 2.83 CI= 1.11–7.19 for 2.46 mg/L vs. 0.36 mg/L of nitrate-N), but only women were analyzed (Weyer et al. 2001). A more recent study from Netherlands did not find an association between bladder cancer and nitrate exposure from drinking water, food and total diet. Those results were not modified by the intake of vitamins C and E and cigarette smoking (Zeegers et al. 2006).

b) Nitrate exposure and breast cancer

Human evidence relating breast cancer and nitrate exposure (or its derivatives) is limited and inconclusive. Few epidemiologic studies are available and most of them with negative or null results.

A case-control study from the US did not show a consistent association between breast cancer and average annual nitrate levels (OR=1.8, 95%CI=0.6-5.0 for ≥1.2 vs. <0.3 mg/L of nitrate-N. Results were limited by the low variation in exposure levels and the short exposure window evaluated (Brody et al. 2006). A Korean case-control study evaluated breast cancer risk and dietary nitrate intake (mean: 421 mg/day for cases and 424 mg/day for controls) relative to antioxidant vitamins intake. Higher risk was observed with higher intake of nitrate/folate (OR = 2.03, 95% CI = 1.16-3.54) (Yang et al. 2010).

Two analyses of breast cancer risk and nitrate exposure are available from the Iowa cohort study. In the first one, no association was found for nitrate in drinking water (Weyer et al. 2001). In a more recent analysis, increased risk of breast cancer was observed among women with higher waterborne ingested nitrate, and folate ingestion of ≥400 μg/day (Inoue-Choi et al. 2012). The lack of data on individual water consumption was a limitation for the exposure assessment in both studies.

c) Nitrate exposure and colorectal cancer

Positive associations were observed in an ecological study (Gulis et al. 2002). Other ecological studies available were focused on CRC mortality, instead of incidence (Kuo et al. 2007; Yang et al. 2007).

The evidence provided by case-control or cohort studies is inconsistent, particularly for levels below the regulatory limit, which is a common scenario in high-income countries. A case-control study from the US found increased risk of colon cancer among subgroups of subjects with nitrate levels > 5mg/L nitrate-N during >10 years. ORs and 95%CI were 2.0 (1.2-3.3) and 2.2 (1.4-3.6) for those with low vitamin C intake and high meat intake,

respectively (De Roos et al. 2003). Another case-control study found 2.9 fold increase risk of proximal colon cancer for levels ≥10 mg/L vs. 0.5 mg/ of nitrate-N, but did not find positive associations for cancer in distal colon or rectum (McElroy et al. 2008). Only one cohort study is available (Weyer et al. 2001). In this study, nitrate in drinking water was not associated with colon cancer, and was inversely associated with rectal cancer.

Regarding dietary nitrate or NOCs: a case-control study showed increased risk of CRC (adenoma) associated with nitrate and nitrite intake from processed meat, regardless of the exposure to other potential carcinogens, such as heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs) (Ward et al. 2007). A cohort study from UK found increased risk of rectal cancer (HR: 1.46; 95% CI: 1.16, 1.84) per 1-SD increase in dietary NDMA intake. Interaction with vitamin C intake was also found (Loh et al. 2011).

d) Nitrate exposure and other cancer outcomes

Nitrate from drinking water was positively associated with stomach cancer risk in ecological studies (Gulis et al. 2002). But no association with nitrate in drinking water or diet was observed in a case-cohort study (van Loon et al. 1998).

Increased risk of Non-Hodgkin Lymphoma (NHL) was observed with ≥4 mg/L vs. <1.6 mg/L nitrate-N levels in a case-control study from Nebraska-US (Ward et al. 1996). Such association was not confirmed in a prospective study (Weyer et al. 2001), or a more recent case-control study from the US (Freedman et al. 2000). In contrast, dietary nitrate (nitrite or NOCs), mainly from animal sources or processed meat, was associated with NHL (subtypes) (Kilfoy et al. 2010).

Nitrate levels in drinking water or diet were not associated either with pancreatic cancer (Aschebrook-Kilfoy et al. 2011; Coss et al. 2004) or brain tumors (glioma) (Dubrow et al. 2010; Michaud et al. 2009; Ward et al. 2005).

Table 1. Nitrate, nitrite and NOCs exposure and cancer outcomes. Summary of the evidence from case-control studies

First author, year of publication, country	Population	Exposure description		Tumors evaluated	Main findings
	Cases/controls	Measure (source and time)	Levels		
(De Roos et al. 2003) USA	376 (colon) 338 (rectum) cases/ 1244 controls	Average levels (drinking water 1960-1987)	Highest levels of nitrate-N in drinking water >5 mg/L during>10 years	Colon Rectum	(+) association OR (95%CI) with colon cancer if ↓ vitamin C intake 2.0 (1.2-3.3) or ↑red meat intake 2.2(1.4-3.6).
(Ward et al. 2007) USA	146/228 cases (colorectal adeno ma)/ / controls (polyp-free)	Average intake of nitrate, nitrite (through meat, FFQ data, measurements)	Highest (1.86–12.28 mg/day) vs. lowest (0 to <0.22 mg/day) nitrate-nitrite levels in meat	Colon Rectum	(+) association, > risk of colon adenoma for nitrate-nitrite intake OR (95%CI): 2.0 (1.0, 3.9). Adjustment for HCA ↓ OR. No interaction with CYP2A6.
(McElroy et al. 2008) USA	475 cases/1447 controls (women)	Average levels in drinking water (exposure in 1990-1992 and 1999-2001)	Highest vs. lowest (≥10 vs. <0.5 mg/L of nitrate-N)	Colon Rectum	(+) association. 2.9 fold ↑ risk for tumors in proximal colon
(Zhu et al. 2014) Canada	1760/2481 cases/controls	Average NMDA intake 1 year before recruitment (FFQ)	Highest vs. lowest quintiles of intake	Colon Rectum	OR (95%CI): 1·42(1·03, 1·96) *p-trend*: 0·005 of CRC, and 1·61 (1·11, 2·35) *p-trend:* 0·01 for rectal carcinoma. Interaction with vitamin E (p=0·017)
(Ward et al. 2003) USA	808/1259 cases/controls	Average levels (drinking water from 1960-1989)	Levels ≥3.09 mg/L vs. <0.6 mg/L (nitrate-N)	Bladder	(-) association. OR (95%CI) of 0.8 (0.4-0.8) among women and 0.5 (0.4-0.8) among men, regardless of vitamin C intake
(Catsburg et al. 2014) USA	1,660/1,586 cases/controls	Intake of nitrate, nitrite, NOCs (processed meats) and Heme	High vs. lowest quintile of intake (≥148.4 vs. ≤64.3 mg/day of nitrate)	Bladder	(+) association with Intake of liver and, salami/pastrami/corned beef, (+) association with Heme intake (nonsmokers).

		through diet (2 years before recruitment)			(+) association with concurrent high intake of nitrate and meat.
(Brody et al. 2006) USA	824 /745 cases/controls (diagnosed between 1988-1995)	Average levels (drinking water from 1972-1995)	Levels ≥1.2 vs. <0.3 mg/L (nitrate-N)	Breast	(+) association (not statistically significant). OR (95%CI): 1.8 (0.6-5.0)
(Yang et al. 2010) Korea	362/362 cases/ controls	Average intake from diet in previous year (FFQ 121 items)	Mean dietary nitrate intake was 422 mg/day Highest vs. lowest nitrate/folate, nitrate/vitamin C, and other ratios	Breast	(+) association OR (95%CI) of 2.03(1.16-3.54) with higher nitrate/folate intake ratio
(Ward et al. 1996) USA	156 cases/ 527 controls	Average levels (community drinking water 1945-1980)	Highest quartile ≥4.0mg/L vs. <1.6 mg/L of nitrate-N	Non-Hodgkin lymphoma	(+) association. OR (95%CI): 2.0 (1.1-3.6).
(Freedman et al. 2000) USA	73 cases (diagnosis in1980-1982)/147 controls	Average levels (community drinking water 1947-1975)	Highest (7.2 mg/L) vs. lowest (0.1 mg/L of nitrate-N).	Non-Hodgkin lymphoma	No association
(Kilfoy et al. 2010) USA	584 cases/710 controls	Average intake of nitrate, nitrite through diet (FFQ 120 items)	>vs.<median intake 112.1 mg/day of nitrate and 1.1 mg/day of nitrite	Non-Hodgkin lymphoma	No association with nitrate (+) association with nitrite from processed meat and animal sources
(Aschebrook-Kilfoy et al. 2013) USA	348/470 cases/controls.	Average intake of nitrate and nitrite (FFQ)	Highest vs. lowest quartile of nitrate (88.3 vs. 22.2) or nitrite intake (0.86 vs. 0.49 mg/1000 kcal)	Non-Hodgkin lymphoma	(+) association with nitrite intake (particularly from animal sources) among women, OR (95%CI):1.9; (1.0-3.4). No association with nitrate intake.

(Coss et al. 2004) USA	189/1,244 cases/controls	Average nitrate levels in water and intake of nitrate, nitrite through diet (FFQ).	Highest vs. lowest quartile levels (>2.8 vs. 0.6 mg/L nitrate-N). Dietary nitrite (>1.3 vs. <0.75 mg/day)	Pancreas	No association for nitrate in water (+) association for >nitrite intake from animal sources. OR (95%CI): 2.3 (1.1- 5.1) for men, 3.2 (1.6- 6.4) for women.
(Ward et al. 2005) USA	130/319 cases/controls (with ≥ 70% of the time with public water supply nitrate levels)	Average intake of nitrate and nitrite through water and diet (FFQ)	Highest vs. lowest quartile levels in water (>4.32 vs. <2.38 mg/L nitrate-N) 1965-1985. Average dietary intake.	Glioma	(+) association suggested with waterborne nitrate No association with dietary nitrite/ nitrate. No interaction with vitamin C.
(Ward et al. 2008) USA	79 stomach cancer, 84 esophagus cáncer cases/ 321 controls	Average nitrate levels in water (1965–1984). Nitrate, nitrite intake through diet (FFQ)	Highest vs. lowest quartile of water nitrate-N levels (>4.35 vs. 2.42 mg/L) and dietary nitrate+nitrite intake (>8.3 vs. <3.8 mg/day)	Stomach Esophagus	(+) association suggested. OR (95%CI):1.2 (0.5-2.7) of stomach and 1.3 (0.6-3.1) of esophagus cancer. Dietary nitrate+nitrite: 1.6 (0.7-3.7) of stomach and 2.2 (0.9-5.7) of esophagus cancer
(Hernández-Ramírez et al. 2009) Mexico	257/478 controls.	Intake of polyphenols, nitrate and nitrite through diet (FFQ)	Highest vs. lowest tertiles (>141.7 vs. ≤90.4 mg/day of nitrate in total diet and >3.9 vs. ≤1.7 mg/day of nitrate in animal sources)	Stomach	(+) association with high nitrate or nitrite intake. >OR for subjects with both low polyphenols and high animal-derived nitrate or nitrite intake. (-) association with polyphenols

FFQ: food frequency questionnaire. OR: Odds ratio. CI: confidence interval. HCA: Heterocyclic amines.

Table2. Nitrate, nitrite and NOCs exposure and cancer outcomes. Summary of the evidence from cohort studies

First author, year of publication, (country)	Population	Exposure description		Tumors evaluated	Main findings
		Exposure measure and time	Levels evaluated		
(Weyer et al. 2001) USA	21,977 women from Iowa (16,541 with municipal water supply)	Average nitrate levels in municipal water (1955-1988)	Highest vs. lowest quartile levels (>2.46 vs.<0.36 mg/L of nitrate-N)	1.Colon 2.Rectum 3.Bladder 4.Breast 5.Others	1.No association 2.Inverse association 3. RR (95%CI) of 2.83(1.1-7.2) women 4. No association 5. (+) association with ovary , (-) with NHL
(Loh et al. 2011) UK	23,363 men and women (3268 cancer cases).	Average intake of NMDA, nitrite through diet (FFQ) Follow up from 1993-1997 to 2008.	Highest vs. lowest quartile of intake of NMDA (>125 vs. <16 ng/day), nitrite (>1.7 vs. <1.2 mg/day)	Colon Rectum	Higher rectal cancer risk. HR (95%CI): 1.46 (1.16, 1.84) per 1-SD increase of NDMA intake Interaction with vitamin C intake
(Dellavalle et al. 2014) China	73,118 women (383, colon cancer and 236 rectal cancer cases).	Average nitrate and nitrite dietary intake (FFQ 77 items) Follow-up 11 years	Highest vs. lowest quintile of nitrate intake (313 vs. 99 mg/1000 kcal).	Colon Rectum	No association with CRC risk HR(95%CI): 1.08 (0.73-1.59). Among women with vitamin C intake <83.9 mg/day and high nitrate intake: 2.45(1.15-5.18; *p-trend*: 0.02). No association with nitrite intake.
(Zeegers et al. 2006) Netherlands	120,852 men and women (4,441 subcohort members with 889 cases were analyzed)	Average nitrate ingestion through water and diet (from 1986, 9.3 years of follow-up)	Highest vs. lowest quintile of NO_3^- intake through water (10.6 vs. 0.5) or food (159 vs. 57 mg/day)	Bladder	No association, regardless of vitamins intake or smoking

(Inoue-Choi et al. 2012) USA	34,388 postmenopausal women (2,875 cases)	Average intake through water and diet (FFQ).	Highest vs. lowest quintile of nitrate-N intake through water (57 vs. 1.6 mg/day) or diet (210 vs. 49 mg/day)	Breast	Higher ORs for women with high waterborne ingested nitrate, and folate ingestion of ≥400 µg/day
(Aschebrook-Kilfoy et al. 2011) USA	303,156 (176,842 men and 126,314 women) with 1,728 cases.	Average intake of nitrate and nitrite through diet (FFQ 124 items. Follow up from 1995-2006	Highest vs. lowest quintile of nitrate+nitrite from processed meat (2.9 vs. 0.15 mg/1000 kcal).	Pancreas	Men with highest nitrate+nitrite intake from processed meat had HR=1.18 (0.95, 1.47) for 10 years follow up, and HR: 1.32 (0.99, 1.76) *p-trend* = 0.11 (exposure at ages 12-13)
(Dubrow et al. 2010) USA	545,770 persons interviewed (585 cases)	Average intake of nitrate, nitrite, processed meat, fruits/vegetables in the previous year (FFQ 24-items). Follow-up 2003.	Highest vs. lowest quintile of nitrate (94.9 vs. 19.4 mg/day), nitrite (0.9 vs. 0.45 mg/day).	Glioma	No association with nitrate or NOCs. No interaction with vitamins intake For nitrite from plant sources HR=1.59 (1.20-2.10*), p-trend = 0.028*.
(Michaud et al. 2009) USA	3 cohorts 49,935 men (HPFS) 92,468 women (NHS I) 95,391 women (NHS II) In total: 335 cases	Intake of meat, nitrate, nitrite, NDMA, and NPYR (FFQ at baseline updated every 4 years). Follow up of HPFS; 1986–2004; NHS I; 1980–2004, NHS II 1991–2005	Highest vs. lowest quintile of intake (specific cut-offs for each cohort)	Glioma	No association RR (95%CI) with processed meat: 0.92 (0.48,1.77) Nitrate: 1.02 (0.66, 1.58), nitrites: 1.26 (0.89, 1.79), or NDMA: 0.88 (0.57, 1.36). No interaction with vitamins C,E or other antioxidant.

(van Loon et al. 1998) Netherlands	20,852 men and women (baseline population). Case-subcohort: 1688 men, 1812 women (282 cases).	Average daily intake of nitrate and nitrite through diet (FFQ) and drinking water (Follow up from 1986 by 6.3 years)	Highest vs. lowest quintile of dietary nitrate (172 vs. 55 mg/day) waterborne NO_3^- (16.5 vs.0.02 mg/day)	Stomach	No association with dietary (RR (95%CI) = 0.80 (0.47-1.37), or waterborne nitrate (RR (95%CI) = 0.88 (0.59-1.32). No interaction with vitamin C.
(Keszei et al. 2013) Netherlands	120,852 men and women. Case-subcohort: 925 cases esophagus-gastric cancer/ 4032 controls	Average daily intake of NOCs, nitrite, Heme (FFQ 150 items). Follow up 16 years (from 1986)	0.1-µg/d increase in intake.	Esophagus Stomach	HR (95%CI): 1.15 (1.05, 1.25) *p*-trend: 0.01 of ESCC, and 1.06 (1.01, 1.10 *p*-trend: 0.09) of GNCA risk in men. ESCC also associated with nitrite and Heme intake.
(Ward et al. 2010) USA	21,977 women (45 cases)	Average nitrate intake from drinking water (1955-1988) and diet (FFQ)	Years consuming water with >5 mg/L of nitrate-N. Highest vs. lowest quartile intake (>41 vs.<17 mg/day)	Thyroid	>5 mg/L nitrate-N for ≥ 5year. RR (95%CI): 2.6(1.1-6.2). For dietary nitrate RR (95%CI): 2.9 (1.0-8.1) *p-trend* =0.046
(Kilfoy et al. 2011) USA	490194 men and women (370 cases)	Average nitrate and nitrite intake through diet in previous year (FFQ 124 items).	Highest vs. lowest quintile of intake (94.8 vs. 19.4 mg/1000 kcal)	Thyroid	(+) association RR (95%CI) = 2.28 (1.29-4.04) and *p-trend* <0.001, similar for papillary and follicular tumors among men. No trend among women. No association with nitrite intake.
(Aschebrook-Kilfoy et al. 2013) China	73,317 women (134 cases)	Average intake of nitrate and nitrite (FFQ). Follow-up 11 years from 1996-2000	Highest vs. lowest quartile of nitrate (251 vs.109) nitrite intake (1.1 vs. 0.6 mg/1000 kcal)	Thyroid	No association for nitrate intake. (+) association for nitrite intake, mainly from processed meats. RR (95%CI): 1.96(1.28-2.99) p-trend <0.01

(Aschebrook-Kilfoy et al. 2012) USA	151 316 women (709 cases)	Average nitrate and nitrite intake (FFQ 124 items). Follow-up 9 years	Highest vs. lowest quintile of nitrate (126.5 vs. 22 mg/1000 kcal) and nitrite (0.9 vs. 0.5 mg/1000 kcal)	Ovarian	(+) association with dietary nitrate HR (95% CI):1.31 (1.01-1.68) and nitrite from animal sources: 1.34 (1.05-1.69).
(Inoue-Choi et al. 2015) USA	28,555 postmenopausal women (315 cases)	Average nitrate levels in water and intake through diet (FFQ) Follow up 1986-2010.	Highest vs. lowest quartile of nitrate-N levels (≥2.98 vs.≤0.47 mg/L).	Ovarian	(+) association. HR(95%CI): 2.03 (1.22-3.38, *p-trend*= 0.003). > risk in private well water users. No interaction with TTHMs. (-) association with dietary nitrate.
(Dellavalle et al. 2013) USA	491,841 men and women (1816 cases)	Average nitrate and nitrite intake (FFQ 124 items). Follow-up 9 years	Highest vs. lowest quintile of nitrate (70.94–864.63 vs. 2.09–24.90 mg/1000 kcal) nitrite intake (0.82–4.00 vs. 0.01–0.52 mg/1000 kcal)	Renal cell carcinoma	(+) association with nitrite intake HR(95%CI):1.28 (1.10–1.49). No association for nitrite from plant sources or nitrate intake.

NO_3^-: Nitrate as ion. HR: Hazard ratio. CI: Confidence interval. CRC: Colorectal cancer. NHL: Non-Hodgkin Lymphoma. NDMA: Nitrosodimethylamine. NPYR: Nitrosopyrolidine. NHS: Nurses' Health Study. HPFS: Health Professionals Follow-Up Study. ESCC: Esophagus squamous cell carcinoma. GNCA: Gastric noncardia adenocarcinoma. TTHMs: Total trihalomethane levels in water.

1.4 Health outcomes evaluated in this thesis

Three frequent tumors (**Figure 7**) among European population were studied in the context of this PhD thesis: bladder, breast and colorectal.

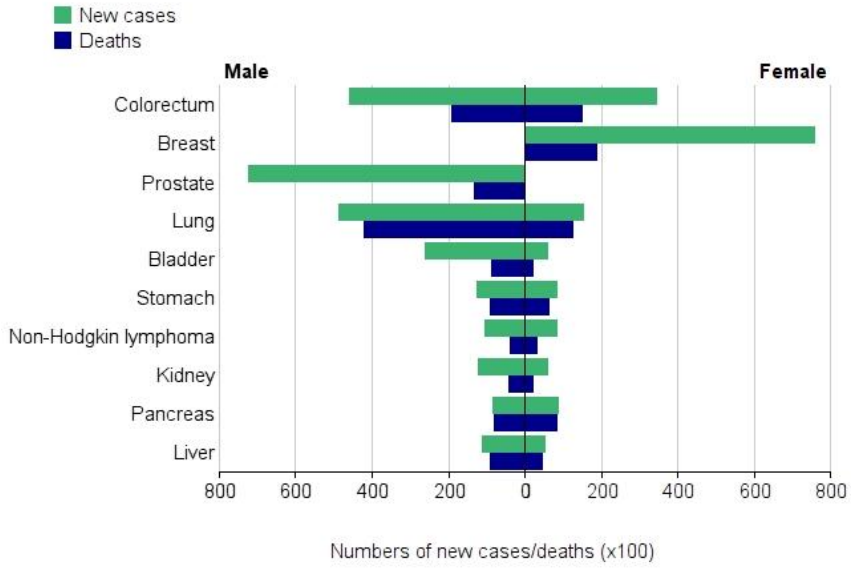


Figure 7. Incidence and mortality rates of the most frequent tumors in Spain and Italy (Source: GLOBOCAN 2012)

1.4.1 Bladder cancer

Bladder cancer is the ninth most frequent cancer worldwide in both sexes, and the 13th most common cause of cancer death. The majority (72%) of the new cases occurred in high-income countries (Stewart 2014). Bladder cancer showed an incidence rate of 13.9 and a mortality rate of 4.0 per 100,000 inhabitants in Spain (Ferlay et al. 2013). Established risk factors include male sex, smoking, exposure to arsenic, and other occupational exposures, such as aromatic amines and polycyclic aromatic hydrocarbons (PAHs) (Kogevinas et al. 2003). Other environmental risk factors have been suggested, including nitrogen products (Marsh et al. 2002) and disinfection by-products (Villanueva et al. 2007).

1.4.2 Breast cancer

Breast cancer is the first cause of cancer mortality and the most incident tumor among women worldwide. In 2012, 1.7 million new cases (25% of all cancers in women) and 0.5 million cancer deaths (15% of the total cancer deaths in women) were diagnosed. (Stewart 2014). In Spain, 25,215 new cases are annually diagnosed (Ferlay et al. 2013) with increasing incidence rates over 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, the use of drugs with estrogenic action, the exposure to ionizing radiation, the family history of breast cancer, previous diagnosis of non-malignant breast diseases and high mamographic density (Hankinson et al. 2004; Stewart 2014). High intake of alcohol or energy are also established risk factors (Romieu et al. 2015). The established risk factors explain only around 50% of the incidence variation of this tumor, and other environmental exposures probably explain a part of the remaining variation (Brody et al. 2007).

1.4.3 Colorectal cancer

Colorectal cancer (CRC) is the third most frequent cancer in men and the second in women, worldwide. More than 1 million new cases and 694,000 deaths are registered annually in both sexes, representing 10% of the global cancer incidence. Although more than 65% of the new cases occur in high income countries, increasing incidence and mortality rates were observed in developing countries during the last decades (Stewart 2014). Established risk factors are red and processed meat intake, (Aune et al. 2013) alcohol intake, physical inactivity and obesity (Larsson and Wolk 2007). In contrast, solid evidence is available for a preventive role of non-steroidal anti-inflammatory drugs (NSAIDs) on the progression from adenoma to carcinoma (Cooper et al. 2010).

2

Rationale

Nitrate is a ubiquitous exposure and frequent contaminant in drinking water. Main routes of human exposure are diet and drinking water ingestion.

Current regulatory levels in drinking water (50 mg/L of NO_3^-) have been established to prevent acute adverse health effects (infant metahemoglobinemia). However, health effects may be produced at lower but long-term exposure levels. Nitrate levels in drinking water may differ widely between countries. Levels below 50 mg/L are frequently observed in high-income countries, with regional variations within countries.

Ingested nitrate leads to the formation of NOCs (N-nitrosamines and N-nitrosamides) which are carcinogens in animals, but their effects in humans are unclear. Based on the current evidence, nitrate is classified as a probable human carcinogen.

Few studies have evaluated the exposure to ingested nitrate as a risk factor for cancer in humans, with contradictory results. The lack of consistency in results is probably related to issues in the study design (e.g. ecologic design), or in the exposure assessment (evaluation of short term exposure windows and lack of data on endogenous nitrosation modulators and water consumption habits).

Studies evaluating long-term exposure to nitrate, and other exposure periods of lifespan, are required, as well as, studies with comprehensive individual information for the exposure assessment, including endogenous nitrosation factors. Such studies would provide valid insights in the potential carcinogenicity of nitrate exposure in humans.

3

Objectives

3.1 General

The main objective of this thesis was to evaluate the potential carcinogenicity of ingested nitrate in humans taking into account endogenous nitrosation modulators and other individual covariates.

3.2 Specifics

a) To describe the current exposure levels in drinking water from municipal distribution system and bottled water in Spain **(Paper I)**.

b) To estimate long-term nitrate levels in drinking water of municipal distribution in municipalities of residence.

c) To evaluate long-term exposure to nitrate in drinking water as a risk factor for bladder cancer **(Paper II)**.

d) To evaluate long-term nitrate exposure through drinking water and diet as a risk factor for:
   - Breast cancer **(Paper III).**
   - Colorectal cancer **(Paper IV).**

4

Methods

"Everything should be made as simple as possible, but not simpler"
Albert Einstein

4.1 Studies design and population

The analyses included in this thesis were conducted in the framework of three large European case-control studies. The general characteristics of these studies and the population recruitment processes are briefly summarized in the following section. Further information is also available in the methods section of the papers comprising this thesis.

4.1.1 The MCC Spain study

The Spanish Multi-case Control study of Cancer (MCC-Spain) is a population-based multicase-control study, conducted between 2008 and 2013 in 12 Spanish regions (**Figure 8**). The study design, the rationale and the population recruitment are fully described in a previous publication (Castaño-Vinyals et al. 2015). The aim of this study was to evaluate the association between environmental exposures and individual factors, including genetic susceptibility, and the occurrence of frequent tumors in Spain. The tumors studied are: female breast, colorectal, prostate, gastric cancer and chronic lymphocytic leukaemia.

Population: Incident cases of the mentioned cancers were recruited from oncological and surgical services in the participating hospitals (n=23), and were histologically confirmed. Cases with ages between 20 and 85 years, residing in the hospital´s catchment areas for at least 6 months prior to recruitment and being able to answer epidemiological questionnaires were included in the study. Population based controls were selected from primary care centers located in the hospitals´ catchment areas. Controls were frequency matched to cases by age, sex and residence area. Controls with previous cancer diagnosis were not included in the study. In total,

10,183 subjects were recruited (4101 controls and 6082 cancer cases), see more details in table 3. The responses rates among cases ranged from 57% to 87%, and among controls from 30% to 77%. The response rates differed by tumor and region (Castaño-Vinyals et al. 2015).

Figure 8. Study areas and institutions participating in the MCC-Spain study

4.1.2 The Spanish Bladder Cancer Study

This was a multicenter, hospital-based case-control study conducted between June 1998 and June 2001. The main objective in this study was to evaluate the exposure to environmental and occupational contaminants and the association with bladder cancer risk. The study included five geographic areas of Spain: Barcelona, Vallès/Bages (including the cities of Sabadell and Manresa), Alicante, Tenerife, and Asturias (**Figure 9**).

Population: Incident cases of bladder cancer were identified through urologic services in the participating hospitals (n=18). All cases were histologically confirmed, aged 20–80 years, and lived in the catchment geographic area of the participating hospitals. Additionally, hospital discharge records, pathology records, and local cancer registries were reviewed in order to complete case

recruitment. Controls were patients admitted to the participating hospitals with diagnoses unrelated to the main risk factors for bladder cancer, such as smoking. Diagnoses of the controls included in the study are detailed in **Table 3**. Cases and controls were individually matched by sex, age group (5-year strata), and geographic area of residence. In total, 1,457 eligible cases and 1,465 eligible controls were identified. Among them, 84% of cases (n = 1,219) and 87% of controls (n = 1,271) provided individual information for the study.

Figure 9. Study areas in Spanish Bladder Cancer Study

4.1.3 The HIWATE Project

HIWATE (Health impacts of long-term exposure to disinfection by-products in drinking water in Europe) started as a major research initiative in Europe to address the limitations of previous research on disinfection by-products (DBPs). The main objective of the project was to investigate potential human health risks (e.g. cancer, reproductive outcomes, pregnancy and birth outcomes, and congenital anomalies) associated with long-term exposure to low levels of disinfectants (such as chlorine) and DBPs occurring in

water for human consumption and used in the food industry. Data on other water contaminants, including nitrate, was also available for epidemiological studies in some regions. The study has made use of existing studies/databases and collected information. The project involved 16 teams in eight European countries. The study protocol, aims and areas included in the HIWATE project are described in a previous publication (Nieuwenhuijsen et al. 2009).

To study the association between colorectal cancer and long-term exposure to DBPs and other water contaminants, a case-control study was launched in Spain and Italy in the framework of the HIWATE project. The Italian areas included in this study were Milan, Pordenone and Udine (**Figure 10**). The Spanish population participating in this project was recruited in Barcelona province, and also participated in the MCC-Spain study, already described.

Figure 10. Study areas of the HIWATE project (Italy)

Population: Incident cases of colorectal cancer were identified in oncological and surgical services of the participating hospitals. Controls were patients admitted to the same hospitals as cases, for acute, non-neoplastic, non-chronic conditions, unrelated to alcohol, tobacco, dietary habits or know risk factors for colorectal cancer. In total 466 cases of colorectal cancr with histological confirmation, and 569 controls were recruited in Milan, Pordenone and Udine. The response rate was 95% among cases and 95% among controls. See more details in **Table 3**.

Tabla 3. Characteristics of the population recruitment in the studies

Study	MCC-Spain	HIWATE (Italy)	SBCS [a]
Total population	*n*=10,183	*n*=1,033	*n*=2,490
Cases	*n*=6,082 incident cancer cases 2,171 CRC [b] 1,750 breast 1,115 prostate 492 stomach esophagus 554 CLL [c]	*n*=466 incident CRC	*n*=1,219 incident bladder cancer
Response rate	57-87% [d]	95%	84 %
Controls	*n*=4,101 Population-based	*n*=569 Hospital-based Diagnoses: Acute surgical conditions (52.2%) Orthopedic diseases (non-trauma) (9%). Trauma (6%) Other diseases (33%)	*n*=1,271 Hospital-based Diagnoses: Hernias (37%) Other abdominal surgery (11%) Fractures (23%) Other orthopedic diseases (7%) Hydrocele (12%) Circulatory diseases (4%) Dermatologic (2%) Ophthalmologic (1%) Other diseases (3%)
Response rate	30-77% [d]	95%	87%
Matching (variables)	Frequency (Age, sex and geographic area of residence)	Frequency (Age, sex and geographic area of residence)	Individual (Age group 5-year strata, sex and geographic area of residence).

[a]SBCS Spanish Bladder Cancer Study. [b]CRC colorectal cancer. [c]CLL chronic lymphocytic leukemia. [d]Response rates differed by tumor and region. More details are provided in (Castaño-Vinyals et al. 2015).

4.2 Data collection

4.2.1 Individual information

The collection of individual data was conducted following similar methods in the MCC-Spain, HIWATE and the Spanish bladder cancer study. In all three studies, individual information was recruited by trained personal in face-to-face interviews, using a computer-assisted questionnaire.

The individual information ascertained included lifelong residential history (full address and the start and stop years of each residence), the main type of water consumed in the residence (municipal, bottled, well/others) and the average amount of water daily intake. Information on water type changes within residences was also collected in a subgroup of population in Spain (n=174 controls), and in Italian population. Sociodemographic characteristics, occupational history, lifelong retrospective environmental exposures, lifestyle factors (e.g smoking habits), medical history and familiar history, were collected among other relevant data. Anthropometric measures and biological samples (blood, urine and saliva) were also collected during the interview. The main characteristics and the main differences on individual data collection methods between studies are summarized in **Table 4.**

In addition, dietary information was collected using validated semi-quantitative food frequency questionnaires (FFQ) (Decarli et al. 1996; Martin-Moreno et al. 1993). The FFQs were either administered during the interview or self-administered and returned by mail (MCC-Spain study). When the FFQ was self-administered, interviewers provided detailed instructions for completing the FFQ.

The questionnaires administered in the MCC-Spain study are available on-line (**http://www.mccspain.org**). More details on the individual information available are provided in the articles that comprise the results of this thesis (see Methods in **papers II, III** and **IV**).

Table 4. Characteristics of the individual data collection in the studies.

Study	Data collection period	Individual data collection instrument	Food frequency questionnaire characteristics
MCC-Spain	2008-2013	Personal interviews	140 food items. Data from 1 year preceding recruitment.
HIWATE (Italy)	2008-2013	Personal interviews (at hospital stay)	78 food items Data from 2 years preceding cancer diagnosis (cases) or hospital admission (controls)
SBCS [a]	1998-2001	Personal interviews (at hospital stay)	78 food items Data from 5 years preceding cancer diagnosis (cases) or hospital admission (controls)

[a] SBCS (Spanish Bladder Cancer Study).

4.2.2 Environmental information

a) Nitrate levels in municipal water

a.1) Current levels- Tap water sampling

Tap water samples were collected between March and July 2010 in 11 Spanish provinces included in the MCC-Spain study. The sampling covered 53 urban and 14 rural municipalities (**Figure 11**). In total, 227 samples were purchased in randomly selected locations (households and public buildings). Sampling procedures followed a common protocol. Samples were analyzed in the Public Health Laboratory of Gipuzkoa–Spain. Levels of nitrate and seven trace elements (arsenic, nickel, chromium, cadmium, lead, selenium and zinc) were measured. Quantification methods and other details may be consulted in the methods section of the **Paper I**.

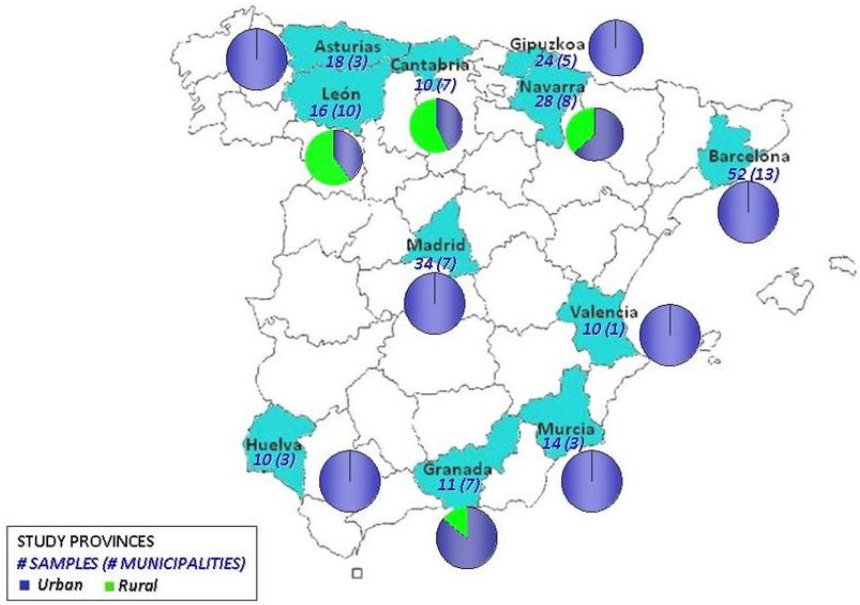


Figure 11. Areas and municipalities of tap water sampling.

a.2) Historical nitrate levels and environmental data

Environmental information was collected in municipalities reported in residential histories, covering at least 80% of the person-years in each study area. A structured questionnaire was sent to water companies and local authorities in the selected municipalities, to collect current and historical environmental information back to 1940. Data requested included nitrate levels in drinking water of public distribution systems and water source (proportion of surface/groundwater). The questionnaire used in the SBCS was originally designed to collect levels of trihalomethanes, and is available in a previous publication (Villanueva et al. 2006). Similar questionnaires were used to collect environmental information in the participating areas of the MCC-Spain study.

Institutions involved in drinking water regulation in the study areas also provided routine monitoring nitrate levels in drinking water. The National Information System in Drinking Water (SINAC) provided nitrate data from 2004 to 2009 (n=7406 measurements) for Spanish areas. The Regional Environmental Health Agency (Milan)

and the Local Health Authority (Pordenone/Udine) provided monitoring nitrate data for Italian areas. Information on water source and characteristics of water distributions systems that was not reported in the questionnaires was obtained through phone calls, e-mails or from official web-sites of local governments, water companies, and other institutions related to drinking water supply in the study areas (e.g. "*Mancomunidades de agua*" in Spain).

b) Nitrate levels in non-municipal water

b.1) Bottled water sampling

The type of water consumed in the most recent (recorded as "current" in the individual questionnaire) and in the longest residence were analyzed among the population recruited in the MCC-Spain study up to 2010. Frequent consumption of bottled water was observed in areas like Barcelona, Murcia and Valencia (**Figure 12**. Unpublished data).

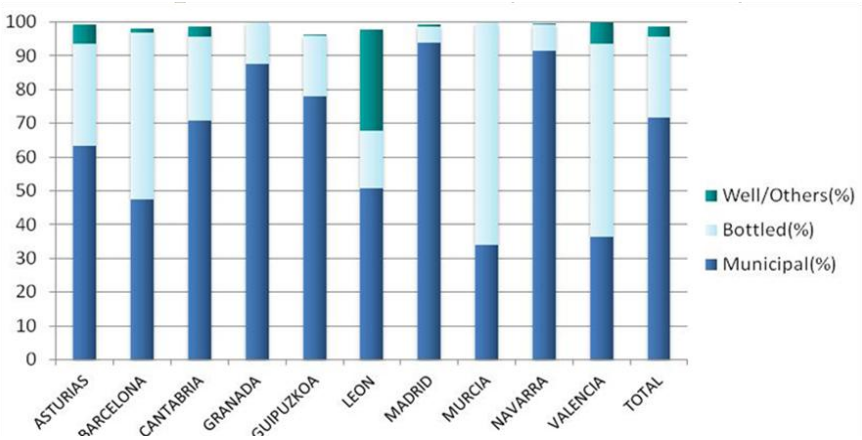


Figure 12. Water type consumed in the current residence among the MCC-Spain areas (n=2974 controls interviewed at 2010).

We aimed to determine the nitrate levels in bottled water brands commercialized in Spain. In January 2011, we purchased 9 samples (500 mL/bottle) of the most consumed brands in Spain, according to the National Association of Bottled Water Companies (ANEABE).

Trace elements were also measured in these samples and the results are detailed in **paper I**. Data from the most consumed bottled water brands in Tenerife and Italy were also available from previous reports and were used for the exposure assessment of bottled water consumers in those areas (Caballero Mesa et al. 2003; D'Alessandro et al. 2012).

b.2) Well water sampling

Among the population recruited in the MCC-Spain study at 2010, controls from León province reported the most frequent well/other water consumption in the current residence (36%) (see **Figure 12**). The frequency was higher when the longest residence was analyzed. Thus, nitrate levels in wells outside the municipal water distribution system, were measured in September 2013 in 21 municipalities of León region.

Controls reporting well/other water consumption in the current residence were contacted by phone calls, and provided the location (complete address) of the well used for water supply in their households. A total of 28 water samples were collected in the identified sampling points (**Figures 13** and **14**). Sampling procedures followed a common protocol. Nitrate, trihalomethanes and trace elements were measured in all samples. The analyses were performed in the Public Health Laboratory of Gipuzkoa–Spain.

Figure 13. A artisan well sampled in León region.

	Municipalities	N
1	Astorga	1
2	Cabreros del Río	1
3	Carrizo	2
4	Cimanes del Tejar	2
5	Cuadros	1
6	El Burgo Ranero	1
7	Fresno de la Vega	1
8	Garrafe de Torio	2
9	La Robla	1
10	Las Omañas	1
11	León	3
12	Mansilla Mayor	2
13	Palacios de Valduerna	1
14	Quintana y Congosto	1
15	Rioseco de Tapia	1
16	San Andrés del Rabanedo	2
17	Valdefresno	1
18	Valverde de la Vírgen	1
19	Vega de Infanzones	1
20	Vegas del Condado	1
21	Villasabariego	1
	Total samples	**28**

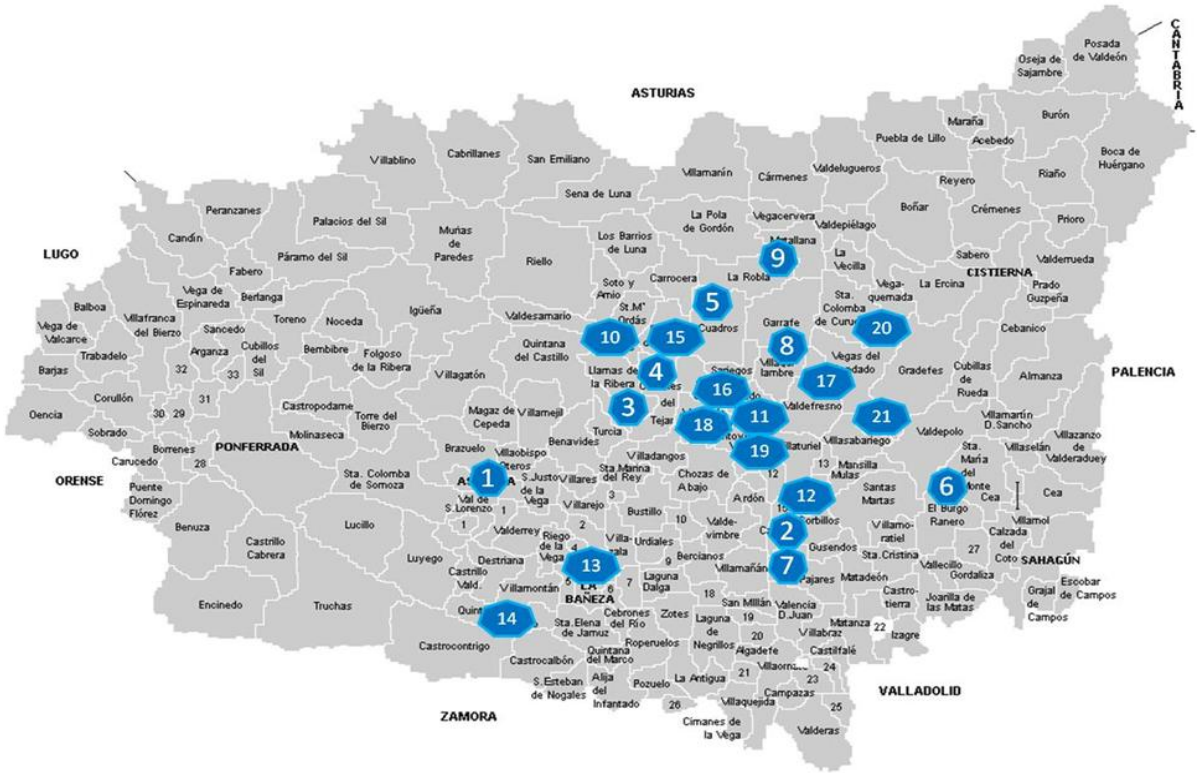


Figure 14. Sampling points of well water in León region.

4.3 Estimation of long-term nitrate levels in drinking water

The aim was to assign annual waterborne exposure levels to the study population, covering a long-term period from age 18 to recruitment. Since the population aged 25 to 85 years, long-term nitrate levels in drinking water were estimated back to 1940 in the study municipalities, considering the particular characteristics of the water distribution system, and the environmental data (actual nitrate measurements) available in each municipality.

4.3.1 Municipalities and water zones definition

The estimation of historical nitrate levels was conducted by water zone, defined as the geographic area supplied by water with similar quality (nitrate levels) and similar water source characteristics (surface/groundwater proportion). In most cases, water zone coincided with municipality, but some municipalities involved multiple water zones. In such cases, if the available levels were similar, historical levels were estimated for the whole municipality to maximize the number of nitrate measurements. Nitrate levels were estimated for multiple water zones in Barcelona city (see **Figure 15**), Usurbil (Gipuzkoa) and Milan (Italy).

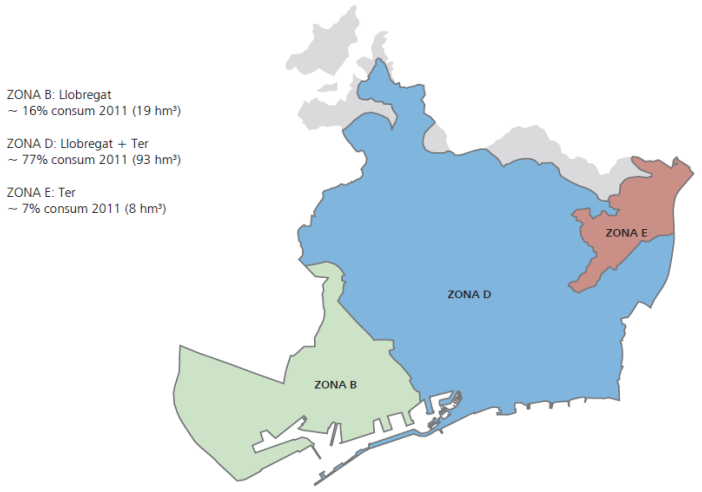


Figure 15. Water zones in Barcelona. Source: *Agència de Salut Pública de Barcelona* (ASPB) report 2012.

4.3.2 Preliminary analysis of available nitrate data in drinking water

Nitrate levels were reported in mg/L of nitrate as ion (NO_3^-). All data available for each water zone (municipality) was compiled in a single database. Previously, the levels reported by different information sources (e.g. water companies and monitoring data) were compared by sampling date and nitrate levels, in order to exclude duplicate measurements in each water zone. The distribution of the available levels was analyzed with histograms and Q-Q plots, and outliers were excluded in each water zone. Nitrate levels observed in samples of tap water collected in 2010 (results of **paper I**) in Spanish municipalities, were similar to historical nitrate levels available (see **Appendix 1**), and were included in the same data base.

Measurements below the quantification limits (QL) (5% of total measurements in Spain) 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).

A brief summary of the environmental data collected in each area is shown in **Table 5**, and more details are shown in **supplemental material** of **papers II** and **IV.**

Table 5. Environmental data available between 1940 and 2010 in the study areas

Study areas (water zones)	Cancer evaluated	Nitrate levels		Water source
		Years with measurements	#Measurements/water zone Median (range)	#Years with data/water zone Median (range)
Spain				
Asturias (17)	BDC,BC,CRC	1979-2009	27.0 (4-867)	71 (13-71)
Barcelona (30)	BDC,BC,CRC	1996-2010	10.5 (2-1078)	60 (22-71)
Cantabria (17)	BC,CRC	1995-2010	14.0 (2-268)	71 (8-71)
Guipuzcoa (34)	BC,CRC	1993-2010	104 (12- 280)	23 (8-51)
León (43)	BC,CRC	1985-2010	8.0 (2-54)	41 (11-71)
Madrid (15)	BC,CRC	1981-2010	13.5 (4-533)	49 (9-71)
Murcia (17)	CRC	1998-2010	14.5 (5-519)	48 (41-66)
Navarra (29)	BC,CRC	1986-2010	28.0 (7-150)	71 (1-71)
Valencia (18)	BC,CRC	1994-2010	44.0 (15-2535)	31 (17-71)
Tenerife (12)	BDC	1999	1(1-1)	71 (71-71)
Italy				
Milan (34)	CRC	1997-2008	10 (7-12)	71 (9-71)
Pordenone-Udine (41)	CRC	1986-2007	9 (3-15)	71 (17-71)

BDC: bladder cancer, BC: breast cancer, CRC: colorectal cancer.

4.3.3 Estimation and imputation of nitrate levels

Annual historical nitrate levels were calculated based on available nitrate measurements in each water zone. For years with nitrate information, measurements were averaged per year. Then, the totality of the measurements available in the water zone were averaged, and this value was imputed to years with missing nitrate levels, as long as percentage of ground water remained stable (changes of ±10%). In the event of wider changes, the percentage of ground water was used to modulate imputed nitrate levels accordingly, assuming that nitrate levels increased at higher percentages of ground water. In water zones where nitrate measurements were not available but percentage of ground water was known (5 water zones from Spain and 1 water zone from Italy) levels were imputed from neighboring municipalities with similar percentage of ground water. In water zones without environmental information (1 water zone from Spain), levels of neighboring municipalities were imputed, regardless the percentage of ground water supplied. **Figure 16** shows the algorithm used for historical nitrate levels estimations and imputations.

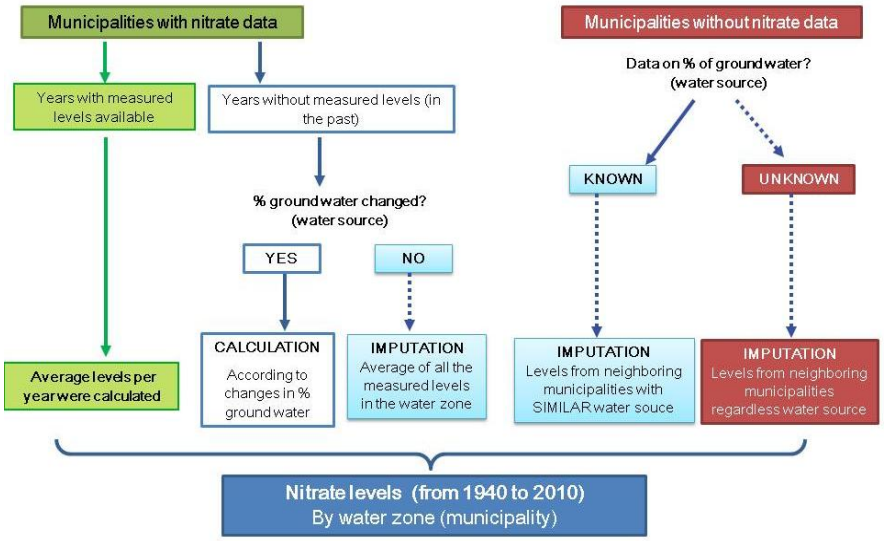


Figure 16. Algorithm applied to estimate historical nitrate levels based on environmental data available. (Discontinuous arrows show imputation routes)

4.3.4 Reliability score for nitrate estimates

We developed an annual score to assess in a semi-quantitative scale the reliability of the estimated nitrate levels. This score was then used for sensitivity analyses.

The score ranged from 0 (lowest reliability) to 2 (highest). A 0 value was directly assigned when nitrate levels were imputed and percentage of ground water (water source) was unknown. A 0.75 value was assigned if the imputed levels were calculated based on <10 measured levels and percentage of groundwater was known. A 0.50 value if the imputed levels were calculated based on ≥10 measured levels and percentage of groundwater was known. An increasing value from 0 to 0.25 was deducted to penalize the time distance of imputed levels from the last year with measured levels. A 1.25 value was assigned when the annual average was based on 1 or 2 nitrate measured levels, and a 2 value when the average was based on >2 measured levels. In summary, we penalized the estimates that were imputed vs. measured, calculated based on less number of measured levels, and more distant in time from a measured level (see table 6).

Table 6. Criteria for assigning reliability score values to nitrate estimates

Nitrate levels	Water source data	Criteria	SCORE value
IMPUTED	IMPUTED		**0.0**
IMPUTED	Available	<10 total measured levels/water zone	**0.50***
IMPUTED	Available	≥10 total measured levels/ water zone	**0.75***
MEASURED	Available	≤ 2 measured levels/year	**1.25**
MEASURED	Available	>2 measured levels/year	**2.0**

*An increasing value between 0.00 and 0.25 was deducted from these values according to time (years) distance from the last MEASURED level.

4.4 Individual exposure assessment

Nitrate levels (including measured and imputed values), surface/groundwater proportions and reliability scores from 1940 to 2010, were linked to lifetime residential histories by municipality of residence and year, in order to obtain long-term annual estimates for each subject.

A main exposure period from age 18 to 2 years before recruitment was defined (adult life exposure). Other exposure periods were also evaluated in the studies included in this thesis (see Methods section of **papers II, III** and **IV**). The number of years with available residential information and the proportion of the residential history that was covered with nitrate levels (measured and imputed), were calculated for the exposure periods evaluated in each study.

4.4.1 Individual exposure indices

Several individual exposure indices were calculated from age 18 to 2 years before recruitment and other exposure periods. The main exposure indices were:

a) Average residential nitrate levels (mg/L)
This variable was the average of nitrate levels assigned to each subject according to the municipalities of residence, during a given time period, and regardless of water consumption habits.

b) Average waterborne ingested nitrate (mg/day)
This variable was estimated based on residential nitrate levels and data on water consumption habits (water type consumed in the residence and amount of water daily intake), in two steps:

First, nitrate levels were assigned year-by-year, according to the water type consumed.

- Residential nitrate levels were assigned to years when tap water consumption was reported.
- For years when bottled consumption was reported: measured values in bottled water brands from Spain (results of **paper I)**

were averaged, using the sales frequency of each brand as a weight (weighted average= 6.1 mg/L), and were assigned to Spanish population. Published values in Italian bottled water (D'Alessandro et al. 2012) were averaged (3.8 mg/L), and this value was assigned to bottled water consumers in Italy.

- For years when well/other water was reported: values observed in the well water sampling in León (range 0.5 to 93 mg/L) were assigned to population from that area, according to the well´s location and the residence addresses (postal code and municipaity). Nitrate levels in wells were not available for other areas, thus missing values were assigned for this analysis.

In a second step, the assigned levels were averaged and multiplied by the daily water intake reported in the interview.

- The water daily intake was computed including the intake of water per-se and other water based beverages, such as coffee, tea and herbal drinks. Overall, the average daily intake in adult life was around 1.0 L/day, and differed slightly between the conducted studies in this thesis (**Table 7**). Water intakes above the 99th percentile (4.0 L/day), considered non plausible, were treated as missing values in the analyses. For these calculations water intake at work and other places was ignored, since water intake at home was the most frequent, according to preliminary observations from four Spanish areas (**Table 8**).

Table 7. Water daily intake at home among study population

Study	Daily water intake (L/day)	
	Cases	Controls
Paper II	0.99 ± 29.9*	1.07 ± 25.6*
Paper III	1.30 ± 0.7†	1.20 ± 0.7†
Paper IV	1.40 ± 0.8†	1.30 ± 0.9†

*mean ± SE. †mean ± SD

Table 8. Proportion of total water daily intake provided by intake at different places among controls recruited at 2011 in four areas of the MCC-Spain study.

Area	N	Intake at: Home mean%(SD)	Work mean %(SD)	Other places mean%(SD)
Barcelona	796	75.3 (28.7)	28.4 (29.7)	0.3 (3.6)
Gipuzkoa	362	75.0 (29.9)	26.3 (30.4)	0.9 (7.1)
Madrid	728	79.2 (27.5)	18.0 (25.8)	2.5 (10.6)
Navarra	274	96.6 (12.9)	2.6 (11.6)	1.8 (9.2)
Total	**2160**	**79.7 (27.7)**	**20.8 (27.9)**	**1.4 (8.2)**

c) Alternative variable of waterborne ingested nitrate (mg/day)

This variable was calculated to address the potential misclassification of the water type consumed (municipal/bottled) in long-lasting residences, and was used in a sensitivity analysis.

A subgroup from Spain with available information on water type changes within residences (n=174 controls) was analyzed. It was observed that 86% of bottled water consumers in the most recent residence (recorded as "current" in the individual questionnaire), actually switched from municipal to bottled water after the year 2000. Similar analyses were done among Italian population, where most of bottled water consumers actually changed from municipal to bottled water after the year 1980 (**Table 9**).

Table 9. Changes of water type among Spanish and Italian population with information available (only controls were analyzed)

	Spain n (%)	Italy n (%)
Population analyzed	174 (100.0)	512 (100)
Changed water type	65 (36.5)	134 (26.2)
Changed at current residence	56 (86.2)*	82 (61.2)*
Year of water type changes Mean (range)	2000 (1959-2008)	1981 (1951-2007)

*Percentage based on population that changed water type.

Based on these observations, an alternative variable for waterborne ingested nitrate was calculated assuming that subjects reporting bottled water consumption and living for at least 10 years in the current (or previous) residence, actually consumed municipal water before the year 2000, and bottled water thereafter. A similar assumption was applied for Italy with the year 1980 as cutoff.

4.5 Estimation of dietary nitrate exposure

The dietary information collected by FFQs included 140 (Spain) or 78 (Italy) food-items. These data were used to estimate the average daily intake of food groups and nutrients (vitamins C, E, D, and energy). Nutrients´ contents were derived from published food composition databases (Farran et al. 2008; Gnagnarella et al. 2004). Nitrate contents were derived from published data (EFSA 2008; Griesenbeck et al. 2009; Jakszyn et al. 2004). If the specific nitrate content was not available for some vegetables (e.g. rocket salad), the information by vegetable group (e.g. green leafy vegetables) was assigned. Nitrate contents (mg/100g) were assigned to 21 vegetables (including tubers), 13 fruits, 17 animal-derived food sources (including red, white, processed meat and dairy products), 3 frequently consumed foodstuff (bread, rice, and pasta), and 1 alcoholic beverage (beer) listed in the FFQs (see more details in **Appendix 2**). Then, nitrate ingestion (mg/day) was estimated according to the nitrate contents assigned (mg/100g) and the ingestion of each food item (g/day) recorded in the FFQs. Nitrate ingestion through vegetable, animal sources and total diet, was estimated. For these calculations "red meat" included: beef, lamb and pork meat, and "Processed meat" included: bacon, hot dogs, ham, Spanish cured ham and other cured sausages.

4.6 Statistical analyses

Similar statistical analyses were conducted in the three case-control studies presented in this thesis. The population analyzed included only subjects with nitrate exposure levels covering ≥70% of the main exposure period: from age 18 to 2 years before recruitment

("adult life") (papers II and III), or the last 30 years before recruitment (paper IV).

4.6.1 Logistic regression analyses

Nitrate exposure levels (either residential or waterborne ingested nitrate) were categorized (see details on Methods of papers II-IV). Unconditional logistic regression was used to estimate odds ratios (OR) and 95% confidence intervals (CI) of the cancers evaluated, for categorized nitrate exposures.

Covariables: Crude models were adjusted for matching variables (sex, age and area of residence). Education was also included in this adjustment in the studies on breast and colorectal cancer (**papers III** and **IV**). Several potential confounders were evaluated en each study. The covariates included: lifestyle factors (smoking, physical activity, body mass index), familial history of cancer, and use of medication, such as non-steriodal anti-inflammatories (NSAIDs), oral contraceptives (OC) and hormonal replacement therapy (HRT) in women. Dietary variables (intake of energy, fiber, alcohol, folate), and endogenous nitrosation modulators (intake of vitamin C, vitamin E, red meat, processed meat, and gastric ulcer history), were also tested as potential confounders.

For selecting confounders, first we conducted bi-variate analyses, and only the variables associated with the outcome were included in the multivariate analyses. These variables were added one by one to models with basic adjustment. Only the variables with conservative *p values* <0.10, and the variables that changed the risk estimates in ≥10% were kept in the model. Final adjusted models included established risk factors for the tumor evaluated (defined a priori), and the covariates selected. These models are detailed in each paper (see Methods of papers II-IV).

Missing values: Multiple imputation methods were applied for the analyses of bladder cancer, given the high percentage of missing information in relevant covariates (see Methods and Appendix. **paper II**). In the studies on breast and colorectal cancer risk (papers

III and IV), missing values were included in the multivariate analyses as a separate category.

Evaluation off effect modifiers and combined effects: Stratified analyses were conducted to evaluate potential effect modifiers (e.g. sex, cancer site). In addition, the models with and without the interaction term were compared using the likelihood ratio test, and *p* values <0.10 were considered indicative of multiplicative interaction. Combined effects of nitrate exposure and endogenous nitrosation modulators were also evaluated.

Sensitivity analyses: Several sensitivity analyses were conducted: using the alternative variable of waterborne ingested nitrate in adult life, excluding subjects with missing values in relevant covariates, and excluding subjects with less reliable interviews or with lower reliability score of the exposure estimates.

The previous analyses were conducted using STATA version 12.0 (Stata Corp, College Station, TX).

4.6.2 Other analyses conducted

Generalized additive models (GAMs) were used to evaluate the exposure-response relationship between nitrate exposure in drinking water and cancer risk. GAMs were obtained for each study area (see results for breast cancer and colorectal cancer risk in **Appendix 3**) and overall areas (see results of **paper IV**).

Meta-smoothing approach was also explored to combine the area-specific exposure-response curves in one single curve. This analysis is systematically applied in multicenter studies on air pollution (Schwartz and Zanobetti 2000) and was explored for the evaluation of CRC risk related to nitrate exposure in drinking water. The results of this analysis were not included in the final manuscript of paper IV since were similar to the results obtained with GAMs. The analysis was conducted in R version 3.1.0 (R Development Core Team 2014, Vienna, Austria; http://R-project.org). More details are available in the **Appendix 4** of this thesis.

Mixed hierarchical regression models with area as random effect were also applied to evaluate the association of nitrate exposure and CRC risk (**paper IV**). In sensitivity analyses, study areas contributing >10% of the CRC cases were excluded from the models to evaluate potential changes in the results. The results of these sensitivity analyses were not included in the final manuscript of paper IV, but are shown in the Appendix section of this thesis (see **Appendix 5**).

5

Results

“Only entropy comes easy”
(Anton Chekhov)

5.1 Paper I

Nitrate and trace elements in municipal and bottled water in Spain.

Key points

- This is one of the few epidemiologic studies analyzing the levels of nitrate in drinking water of public distribution covering several regions of Spain: 11 provinces comprising 67 municipalities.
- Nitrate levels were below the maximum regulatory limits (50mg/L of NO_3^-) and differed among regions.
- Nitrate was evaluated in samples of the most consumed brands of bottled water, and showed low levels.
- Trace elements were also analyzed in drinking water (both tap and bottled water) and resulted unquantifiable.

Espejo-Herrera N, Kogevinas M, Castaño-Vinyals G, Aragonés N, Boldo E, Ardanaz E, Azpiroz L, Ulibarrena E, Tardón A, Molina AJ, López-Rojo C, Jiménez-Moleón JJ, Capelo R, Gómez-Acebo I, Ripoll M, Villanueva CM; Multicase Control Study of Cancer (MCC)-Spain Water Working Group. **Nitrate and trace elements in municipal and bottled water in Spain**. Gac Sanit. 2013;27(2):156-60.

doi:10.1016/j.gaceta.2012.02.002

5.2 Paper II

Nitrate in drinking water and bladder cancer risk in Spain.

Key points

- This case-control study was conducted to evaluate the exposure to nitrate in drinking water as a potential risk factor for bladder cancer.

- Mean long-term residential nitrate levels ranged from 2.1 mg/L to 12.0 mg/L of NO_3^- among regions (Asturias, Barcelona, Vallès-Bages, and Tenerife).

- Increased risk of bladder cancer was observed only among subjects with longest exposure duration (>20 years) to the highest levels in the residence (>9.5 mg/L).

- Ingested nitrate through drinking water, calculated based on individual water consumption habits led to non-statistically significant inverse associations, probably confounded by the protective effect of water intake on bladder cancer.

- Endogenous nitrosation factors (e.g. vitamin C intake) and trihalomethanes levels in drinking water did not modify the results.

Espejo-Herrera N, Cantor KP, Malats N, Silverman DT, Tardón A, García-Closas R, Serra C, Kogevinas M, Villanueva CM. **Nitrate in drinking water and bladder cancer risk in Spain**. Environ Res. 2015; 137: 299-307.

doi:10.1016/j.envres.2014.10.034

5.3 Paper III

Ingested nitrate and breast cancer risk in the Spanish Multicase-Control study on Cancer (MCC-Spain) *

Key points

- This is the first case-control study evaluating breast cancer and ingested nitrate conducted among European population, including pre and postmenopausal women.

- Average (mean±SD) waterborne ingested nitrate levels in adult life were 6.4±6.8 mg/day among the women analyzed.

- Statistically significant increased risk of breast cancer was found only among postmenopausal women with both highest waterborne nitrate and highest red or processed meat intake.

- Dietary ingested nitrate (mean±SD: 125±81.4 mg/day) was not associated with breast cancer risk among pre or postmenopausal women, regardless of the vegetable or animal source.

*Under revision on Environmental Health Perspectives
(Submission date: June 12th, 2015)
This paper is reproduced according to the original submitted version.

Espejo-Herrera N, Gracia-Lavedan E, Pollan M, Aragonés N, Boldo E, Perez-Gomez B, Altzibar JM, Amiano P, Zabala AJ, Ardanaz E, Guevara M, Molina AJ, Barrio JP, Gómez-Acebo I, Tardón A, Peiró R, Chirlaque MD, Palau M, Muñoz M, Font-Ribera L, Castaño-Vinyals G, Kogevinas M, Villanueva CM. Ingested Nitrate and Breast Cancer in the Spanish Multicase-Control Study on Cancer (MCC-Spain). Environ Health Perspect. 2016 Mar 4.

doi:10.1289/ehp.1510334

5.4 Paper IV

Colorectal cancer risk and nitrate exposure through drinking water and diet*

Key points

- This study compiled two large European case-control studies on colorectal cancer and water contaminants and was conducted to evaluate the risk of colorectal cancer (CRC) associated with nitrate in drinking water and diet

- Average waterborne ingested nitrate ranged from 3.4 to 19.7 mg/day, among the study areas (9 areas from Spain and 2 from Italy).

- Results suggest a positive association between CRC risk and long-term exposure to nitrate in drinking water, at levels below the current regulatory limit in Europe (50 mg/L of NO_3^-).

- Strongest associations were found among men compared to women, and among a subgroup of men with high meat intake.

- Ingested nitrate from total dietary sources led to null associations. However, nitrate from animal sources was positively associated with rectal cancer.

*Original manuscript to be submitted for publication

Espejo-Herrera N, Gràcia-Lavedan E, Boldo E, Aragonés N, Pérez-Gómez B, Pollán M, Molina AJ, Fernández T, Martín V, La Vecchia C, Bosetti C, Tavani A, Polesel J, Serraino D, Gómez Acebo I, Altzibar JM, Ardanaz E, Burgui R, Pisa F, Fernández-Tardón G, Tardón A, Peiró R, Navarro C, Castaño-Vinyals G, Moreno V, Righi E, Aggazzotti G, Basagaña X, Nieuwenhuijsen M, Kogevinas M, Villanueva CM. Colorectal cancer risk and nitrate exposure through drinking water and diet. Int J Cancer. 2016 Jul 15;139(2):334-46. doi: 10.1002/ijc.30083

6

Discussion

"Ever tried. Ever failed.
No matter. Try again.
Fail again. Fail better"
(Samuel Beckett)

This section includes an overall discussion on the main findings, cross-sectional methodological issues and additional comments on the results presented in this thesis. Interpretation of the results and further discussion on specific aspects are stated in the discussion section of each paper.

6.1 Main findings

The first paper of this thesis is one of the few epidemiologic studies presenting a systematic analysis of nitrate levels in drinking water of public distribution, including several geographical areas in Spain. Nitrate levels in municipal water from 67 municipalities (11 provinces) resulted below the maximum regulatory limit in Europe (50mg/L of NO_3^-) (median: 4.2 mg/L range: <0.1-29.0 mg/L), and differed among regions. Similar levels were found in urban and rural municipalities from some Spanish areas (geometric mean=4.2 vs. 4.4 mg/L, respectively). Low nitrate levels were also found in samples of the most consumed bottled water brands (median: 5.2 mg/L range: 2.3-15.6 mg/L). Levels observed in some brands of bottled water were higher than levels in municipal water from some regions. Tap and bottled water showed unquantifiable levels of trace elements (arsenic, cadmium, chromium, lead, nickel, selenium and zinc). These results showed the current nitrate exposure levels in drinking water, among the Spanish population, and were used for the long-term exposure assessment in the case-control studies included in this thesis.

The papers II, III and IV provide evidence from large European case-controls studies that evaluated the association between three highly prevalent tumors and nitrate exposure through drinking water and/or diet.

In the **paper II**, focused on bladder cancer, average long-term (adult life) residential nitrate levels ranged from 2.1 to 12.0 mg/L of NO_3^- among regions (Asturias, Barcelona, Vallès-Bages, and Tenerife). Increased risk of bladder cancer was observed only among subjects exposed to residential nitrate levels >9.5 mg/L during >20 years. Waterborne ingested nitrate led to non-significant inverse associations, probably confounded by the protective effect of water intake on bladder cancer. Endogenous nitrosation factors (e.g. vitamin C intake) did not modify the results.

In the **paper III**, evaluating breast cancer risk, average (mean±SD) waterborne ingested nitrate in adult life ranged from 3.2±2.9 to 13.5±7.5 mg/day, among eight Spanish regions. Higher risk of breast cancer was found among postmenopausal women with simultaneous high intake of waterborne nitrate and red or processed meat. Average ingested nitrate through diet ranged from 88±48.7 to 154±87.8 mg/day, among regions. These levels were not associated with breast cancer risk, neither among pre or postmenopausal women, regardless of the vegetable or animal source.

In the **paper IV**, evaluating colorectal cancer risk, average waterborne ingested nitrate during the last 30 years before recruitment, ranged from 3.4±3.3 to 19.7±22.6 mg/day, among 11 areas from Spain and Italy. Results of logistic regression and other analyses suggested a positive association between colorectal cancer risk and exposure to nitrate through drinking water, particularly among men compared to women (*p value for interaction<0.009*), and among men with simultaneous high meat intake. Ingested nitrate through diet ranged from 78.1±48.6 to 154±68.4 mg/day and led to null associations, overall. However, nitrate intake from animal sources was positively associated with rectal cancer, among overall population.

6.2 Methodological issues

6.2.1 Study Design and population

The case-control design is preferred for cancer studies, since allows the simultaneous evaluation of multiple exposures for one single outcome, as well as the evaluation of interactions (Breslow and Day 1980). However, case-control studies are susceptible to bias, especially selection bias that may reduce the comparability between cases and controls. The most important step to avoid this bias is an adequate selection of controls, which is particularly challenging in case-control studies on environmental exposures (Agudo and González 1999). The recruitment of population-based controls would reduce potential selection bias, compared to hospital-based controls, although both alternatives for selecting controls may have inconvenients.

In the studies including hospital-based controls (paper II and paper IV Italy) strict recruitment criteria were applied to maximize comparability between cases and controls (Nieuwenhuijsen et al. 2009; Villanueva et al. 2007). Only subjects with acute, non-neoplastic, non-chronic diseases and unrelated to alcohol, tobacco, dietary habits or known cancer risk factors were selected as controls (see specific diagnoses in **Table 3**). Since controls were selected from the same hospitals than cases and matched by residence area, overmatching may not be ruled out. The admission to a hospital correlates to the place of residence and the exposure to environmental factors. Matching by a factor related to the exposure but unrelated to the disease, leads to overmatching and may produce valid, but imprecise estimations (Agudo and González 1999).

The main inconvenient when population-based controls are selected is the potential low response rate. Low response rates are a threat for external validity. In the MCC-Spain study, population based controls were selected using a previously validated method in Spanish population (Castaño-Vinyals et al. 2011) that improved the participation rate. Although the overall response rate among controls was relatively low (53%), the rates differed by region (range: 30%- 77%), and were higher among the regions analyzed in these studies (Castaño-Vinyals et al. 2015).

6.2.2 Exposure Assessment

a) Issues on individual data collection

In the collection of individual data, information bias is the main concern. However, potential information bias was carefully addressed in these studies. The individual information was collected by trained interviewers, using a computer-assisted standardized questionnaire. In addition, interview quality was included in the multivariate analyses as a potential confounder, as previous studies recommended (Villanueva et al. 2009), and the adjustment for this variable strengthened the associations between breast cancer and nitrate exposure (**paper III**). Information on interview quality was also used in sensitivity analyses. Subjects with lower quality interview were excluded, and ORs obtained were slightly higher, although the differences were not statistically significant, for all tumors evaluated.

The probability of recall bias was low, since the questions on residential history and water consumption habits, the most relevant for the exposure assessment, were collected among a pool of several questions. Thus, patients were not able to relate their diagnosis to any specific exposure from the questionnaire.

b) Assessment of exposure to nitrate in drinking water

The scarcity of monitoring nitrate data in the study municipalities is a main limitation of the exposure assessment in these studies, as it was discussed in papers II, III and IV. Exposure assessment to water contaminants in case-control studies on cancer is challenging, particularly when limited historical exposure measurements are available. Monitoring nitrate levels were available only after 1980 in the Spanish study municipalities, and systematic reports were available since 2003, after the establishment of the SINAC (*Sistema de Información Nacional en Aguas de Consumo*). In Italy, only monitoring levels were available, provided by water regulatory institutions, between 1986 and 2008. In addition, the frequency of nitrate measurement in drinking water depends on the municipalities´ population size in Spain (SINAC 2011). Thus, less

data was available in rural municipalities that are more susceptible to nitrate contamination from agriculture activities.

Since the number of years with actual nitrate measurements available in the study municipalities was insufficient for a long-term exposure evaluation, missing levels were estimated an imputed under some assumptions. The use of assumptions and imputations may lead to exposure measurement error and exposure misclassification. To address this, a reliability score for the exposure estimates (residential nitrate levels) was designed and used in sensitivity analyses. After excluding the estimates with lower reliability score, the results obtained were similar to the main results, overall. This score has limitations since the values were arbitrarily defined, and low quality estimates were mainly observed in areas with highest exposure levels. In the **paper IV**, a shorter exposure period was evaluated (last 30 years before recruitment) to reduce the use of imputed or estimated levels in the analyses of CRC risk and the potential measurement error. Although measurement errors in the exposure are not differential, may attenuate the associations toward the null, as it was observed in previous case-control studies (Villanueva et al. 2006). Therefore, more strategies to address measurement error and potential exposure misclassification are required in studies on water contaminants and cancer outcomes, particularly when actual exposure measurements are not sufficient to evaluate long-term exposure periods.

The estimation and imputation of historical nitrate levels was also hampered by the insufficient information on environmental determinants of nitrate levels in drinking water per municipality. To estimate historical nitrate levels the proportion of ground water was used as a main predictor, under the assumption that nitrate levels were higher at higher proportions of ground water. However, high variability of nitrate levels may be observed in ground water sources, according to the characteristics of aquifers and soils (Aschebrook-Kilfoy et al. 2012). For example, shallow unconfined aquifers are more susceptible to nitrate contamination in agricultural areas (Burkart and Stoner 2007). Other relevant factors should also be accounted to estimate nitrate levels in drinking water, including the use of fertilizers, land use pattern and sewage or waste water accumulation in urban areas (Wakida and Lerner 2005). However,

these data were not collected from local governments, since the questionnaire used was not specifically addressed to estimate nitrate exposure. Some data on fertilizers use is available on-line from the Ministry of Agriculture in Spain, but only national and regional statistics are available for recent years (from 2005 onwards) (MARM 2015).

Alternative methods for estimating historical nitrate levels are required, particularly in settings where such information is scarce. Land-use regression (LUR) models seems to be a promising alternative to estimate nitrate contamination in surface and groundwater sources, as it was shown previously (Messier et al. 2014). These models are particularly convenient for the estimation of nitrate levels in water sources out of the municipal distribution system. However, a large amount of additional environmental data (e.g. use of farm and non-farm fertilizers, manure, atmospheric deposition of NO_3-, population density, etc) is required for modelling. In addition, the accuracy of LUR models relays on the amount of actual monitoring nitrate levels available in a given area (Messier et al. 2014). This emphasizes the need of more monitoring data on nitrate in drinking water to improve exposure assessment in cancer studies. Other models have been developed for estimation of nitrate levels in private wells (Nolan and Hitt 2006; Wheeler et al. 2015), since these water sources are more prone to nitrate contamination, and levels are not systematically measured by the governments. Some efforts should be done in future studies to apply these models in Europe and other contexts, especially in regions where private well water consumption is frequent.

c) Assessment of individual exposure indices

Two main individual exposure indices were used in these studies: waterborne ingested nitrate (papers II, III and IV) and residential levels (papers II and IV). The potential sources of errors for both indices are discussed below.

For calculations of waterborne ingested nitrate levels, specific exposure levels were assigned according to the wayer type reported in each residence, and were multiplied by the amount of water intake (individual data). Water intake at work and other places were

ignored in these calculations, since most of the total daily intake among this population was supplied by the intake at home (see **Table 8**). However, this may be a limitation in these studies, since the proportion of water intake at work and other places may differ between regions or countries, and may change in time. In future studies, specific water consumption habits should be analyzed and water intake at work and other places should be considered in the exposure assessment if it is required.

The estimation of waterborne ingested nitrate levels was also prone to error because an average nitrate level of published data was assigned for subjects reporting bottled water consumption, ignoring the potential variability of nitrate levels between bottled water brands and regions. Nitrate levels in well water were not available for all the study areas, which probably introduced some non-differential measurement error, and underestimates waterborne nitrate intake among subjects with long-lasting well water consumption. However, the consumption of well water was less frequent in other areas, as well as the time consuming this water type, compared to bottled or municipal water (see more details in **Appendix 6**). Subjects reported only one water type by residence, and changes on water type consumed in long-lasting residences were not recruited among the totality of the study population. This may be another source of measurement error that was addressed by the calculation of an alternative variable, as it is described in the **Methods** section of this thesis (**page 45**) and in each paper. The water type used for cooking, and the amount of cooked water intake were not accounted for the calculations of waterborne ingested nitrate, probably leading to underestimation of the ingestion levels. Finally, the amount of water intake was collected retrospectively, thus errors in reporting this information may not be ruled out.

In other hand, average residential nitrate levels were estimated for each municipality (water zone), and were assigned as individual exposure levels to each subject, leading to Berkson type error (Heid et al. 2004). However, the results of these analyses are useful to identify the potential risk associated with water ingestion sources that were not included in the calculation of individual water intake (e.g. water used for cooking). Potential measurement error due to the use of imputed vs. measured levels was already discussed.

d) Assessment of dietary nitrate

Exposure assessment to dietary nitrate was based on published information on nitrate contents in food products, and self-reported dietary information collected through FFQs (see **Appendix 2**)

One of the main concerns relays on the lack of updated food composition tables on nitrate and its derivatives. The food composition tables used for dietary nitrate estimations in this thesis, were designed in 2004, based on studies available from 1970 onwards, and were not country specific (Jakszyn et al. 2004), thus may lead to measurement errors.

Another concern relays on the information provided by the FFQs. Although FFQs are valid tools for collecting dietary information in epidemiologic studies, they have some limitations that were already discussed in papers III and IV, but some more general comments are still required. Specific information in some relevant vegetable sources of dietary nitrate was not available and was extrapolated from levels reported for the vegetable group. Data on processing methods of vegetables (e.g storing, peeling, cooking), was not available either. Processing methods may reduce nitrate content in vegetables and should be accounted in the calculations of dietary nitrate intake. The lack of these data was expected, since the FFQs applied were not specifically aimed to estimate nitrate intake.

Finally, nitrate content in food products, in particular in vegetables, depends on other environmental factors that were not accounted in these studies. Those factors, such as the amount of fertilizers used in agriculture (EFSA 2008), may have geographical and time variation, and should be considered in the estimation of dietary nitrate, to improve the exposure assessment in future studies.

6.2.3 Issues in statistical analyses

Although these analyses were conducted in the framework of large case-control studies, the exposure levels were categorized (tertiles, quartiles and others), probably reducing the statistical power of results. The analysis of the continuous exposures was not feasible,

since normality could not be achieved with the transformation methods available. Statistical power is concerning in stratified analyses, given the low number of subjects observed in some categories, particularly among the groups with highest nitrate exposure levels.

Average nitrate exposure levels in adult life (and other exposure periods) were used to estimate the risk of cancer, similarly to other studies. However, the use of average levels may hide exposure periods with higher or lower levels of contaminants that were not easy to evaluate given the limited data available. Other exposure variables were calculated, such as the number of years with residential nitrate levels above a given threshold (e.g. 75^{th} percentile of levels observed among controls), and higher OR were found in the analysis of bladder cancer (paper II). However, the results for other tumors (papers III and IV) were similar to those observed with the average levels. The use of alternative exposure measures, other than the average level, may affect the risk estimates and should be evaluated in epidemiologic studies, in particular when long-term exposure periods are under evaluation.

Wide differences in nitrate levels among areas hampered the statistical analyses. In addition, the levels were polarized, with lowest exposures in Asturias and Madrid, and highest in Barcelona and Italian regions. To address this, several alternatives for categorization were explored in the logistic regression analyses, and the cutoffs were finally defined attempting to have representation of all study areas in each category. A more homogeneous distribution was observed for waterborne ingested nitrate levels compared to residential levels. Mixed models with area as random effects were also applied for the evaluation of CRC risk (paper IV). The results of those models were similar to those observed with logistic regression. Sensitivity analyses were conducted with residential nitrate levels, using mixed models. In these analyses, areas with >10% of cases were alternatively excluded from the models. Lowest ORs were found after excluding Barcelona, showing that the results were driven by this area. However, the associations observed remained positive and statistically significant even after excluding Barcelona from the analysis (see **Appendix 5**). In addition to the wide differences in nitrate levels among areas, the distribution of cases and controls according to the exposure levels was

heterogeneous among areas. An unexpected high density of controls was observed for highest residential nitrate levels (>30 mg/L of NO_3^-) in Milan, differing from the distribution in other areas (see **Appendix 7**).

Heterogeneity of exposure-response relationships among the study areas was another concern for statistical analyses. This issue was addressed by applying Generalized Additive Models (GAMs) and meta-smoothing analyses in the evaluation of colorecatal cancer risk and nitrate exposure (paper IV). Although these analyses were also limited by the low number of areas with highest exposure levels, were useful to obtain one single exposure-response curve for the totality of areas. Only results of GAMs were included in the final manuscript of the paper IV, since both methods led comparable results, consistent with results of studies on air pollutants (Samoli et al. 2003). Additional information is available in the Paper IV and in **Appendix 4** of this thesis.

6.2.4 Residual confounding

Intake of nitrate in drinking water is highly correlated with the amount of water intake (*Spearman correlation coefficient (r): 0.70*). Regarding bladder cancer risk, it is difficult to disentangle the potential carcinogenic effect of nitrate from the protective effect of water intake reported in previous studies (Michaud et al. 2007), thus residual negative confounding cannot be ruled out.

The protective effect of nitrate from vegetable sources may be confounded by the protective effect of vegetables intake on CRC and other cancers (Bradbury et al. 2014), although more recent evidence do not support inverse associations between CRC and vegetable intake (Leenders et al. 2015). Similarly, the increased cancer risk observed for nitrate from animal sources may be confounded by red and processed meat intake, although a recent meta-analysis found weak associations with no clear dose-response patterns for red meat intake and CRC (Alexander et al. 2015)

6.3 Debate on potential beneficial effects of ingested nitrate

Increasing evidence on potential beneficial effects of dietary nitrate exposure opened a debate (Sindelar and Milkowski 2012). Although the evaluation of potential beneficial effects was beyond the scope of thesis, protective associations were observed with ingested nitrate from dietary vegetable sources. These results were consistent for two of the tumors evaluated (breast and colorectal cancer), and with results of a recent prospective study that found protective associations between nitrate ingestion from plant sources and colorectal cancer risk, although the results were not statistically significant (Dellavalle et al. 2014).

Beneficial effects of ingested nitrate would be mediated by the endogenous formation of nitric oxide (NO) via nitrate-nitrite-nitric oxide pathway (Lundberg et al. 2008). This metabolic pathway is known as the “mammalian nitrogen oxide cycle" and shares some steps with NOCs formation (Lundberg et al. 2009). NO may be pro or anti-apoptotic for some cells, depending on the rate of NO production and the interaction with other molecules (e.g. iron, proteins, and reactive oxygen species). Long-lasting production of NO is a pro-apoptotic modulator, but low or physiological concentrations of NO prevent cells from apoptosis (Chung et al. 2001). The effects produced by NO may interplay with carcinogenic mechanisms attributed to NOCs, but more clinical, laboratory and epidemiological investigations are required to better understand the complexity of ingested nitrate in the human body.

6.4 Contributions and main strengths of this thesis

This thesis provides the results of a large descriptive study of nitrate levels in drinking water, conducted for the first time in several regions of Spain. The current nitrate levels in municipal and bottled drinking water from Spain resulted below the regulatory limit in EU. For municipal nitrate levels, high variability was observed between regions, but no differences were found between urban and rural municipalities analyzed. Nitrate levels in bottled water differed widely between brands, and levels in some brands were

higher than in levels municipal water from some areas (Gipuzkoa and Asturias).

This thesis also presents results of the first case-control studies conducted among European population to evaluate carcinogenic effects of nitrate exposure through drinking water and diet. In the context of these case-control studies on cancer: historical nitrate levels (from 1940 to 2010) in municipal drinking water were estimated for 12 Spanish and 3 Italian areas, comprising 307 water zones, despite the limited number of measurements available for each municipality (or water zone). Average levels between 1940 and 2010 were similar to the levels observed in municipal water samples in 2010, and were below the current regulatory limit, overall areas (**Appendix 1**). Individual exposure to nitrate through drinking water and diet was estimated among a large group of European population. Average residential and waterborne ingested nitrate levels in drinking water were calculated for adult life and ranged from 1.6 to 30.0 mg/L (of NO_3^{-}), and from 2.9 to 22.6 mg/day, respectively, and were lower than those reported in studies from other countries.

The results of case-control studies suggest that waterborne nitrate exposure, at levels below the current regulatory limit, is positively associated with cancer risk, particularly among subgroups of the population with high simultaneous exposure to other potential risk factors. For bladder cancer, highest risk was found among subjects with high residential nitrate levels (>9.5 mg/L) during >20 years, and among subjects with simultaneous high trihalomethane levels in residential drinking water. For breast cancer: highest risk was found among post-menopausal women with simultaneous high waterborne nitrate and high red or processed meat intake. And for colorectal cancer: highest risk was found among men with simultaneous high waterborne nitrate and red or processed meat intake.

The individual intake of nitrate through diet was also estimated for this population, and was similar to reports from other western countries. These levels and ingestion levels from vegetable sources were inversely associated with breast and colorectal cancer, although the associations were not statistically significant. In contrast, nitrate ingestion from animal sources was positively associated with rectal cancer risk.

In summary, these results suggest that carcinogenic effects of nitrate may depend on the ingestion route (drinking water, animal or vegetable dietary sources). Nitrate exposure through drinking water, at levels below the current regulatory limit, may increase cancer risk. These results did not change after the adjustment for dietary nitrate through different ingestion sources (**Appendix 8**). In contrast, dietary nitrate was not associated with cancer risk. Moreover, the results suggested inverse associations for vegetable derived nitrate. The mechanisms underlying these observations warrant further research. These results must be confirmed in future studies, and should be accounted in upcoming revisions of water quality guidelines.

A main strength of these analyses was the availability of comprehensive individual information for the exposure assessment. The detailed lifetime residential information, and the information on water type consumed in each residence, allowed the assignment of specific long-term nitrate exposure levels for the population analyzed. In addition, individual information on amount of water daily intake was available to estimate waterborne nitrate ingestion for each subject. The collected individual information also allowed the evaluation of several potential confounders and effect modifiers that were not evaluated in some previous studies. These data included: occupational history (paper II) and endogenous nitrosation factors (papers II-IV). The potential confounding and effect modification by trihalomethane levels were analyzed in the association of nitrate exposure and bladder cancer risk (**paper II**), and was explored in additional analyses for CRC risk (see results in **Appendix 9**).

6.5 Implications for future research on nitrate exposure and cancer effects

The evidence on nitrate exposure at levels under regulatory limits and cancer effects remains unclear. It is important to carry on the research of these effects given the potential increasing human exposure to nitrate in the future decades, due to climate change (Stuart et al. 2011). The large studies presented in this thesis support the potential carcinogenicity of ingested nitrate, mainly

under specific individual conditions. More studies based on individual exposure are needed to confirm these results and to increase the available evidence. Few case-control and cohort studies are available for different cancer outcomes (**Table 1**). These studies are insufficient to conduct meta-analysis that may provide more conclusive results. To date, only one meta-analysis has been conducted on nitrate exposure through drinking water and bladder cancer risk, but the results were not conclusive, due to the limited studies available (two cohorts, two case-controls, and one ecological study) (Wang et al. 2012).

As it was stated previously in the Work Group Report on nitrate on drinking water nitrate and health (Ward et al. 2005), more studies with an improved characterization of long-term exposures are still needed, particularly in regions where few analyses have been conducted, including Europe and developing countries. For this purpose, the collection and the accessibility of monitoring data on water contaminants must be enhanced. The conduction of case-control or cohort studies in settings with more available environmental data must be encouraged. At the same time, more studies applying geographical information systems (and other novel approaches for the estimation of nitrate levels in drinking water) should be conducted to validate their use in studies on water contaminants and cancer.

The current non-conclusive evidence on nitrate exposure and cancer outcomes may be clarified with the investigation of other potential confounders or effect modifiers, not analyzed in the context of this thesis. Additional factors related to endogenous nitrosation, such as the intake of Heme (Bastide et al. 2011) or polyphenols (Hernández-Ramírez et al. 2009), should be further investigated. Heme is ingested through red and processed meat and catalyzes the endogenous formation of apparent total N-nitroso compounds, probably increasing the risk of cancer associated with ingested nitrate. In contrast, polyphenols are inhibitors of endogenous nitrosation, and may reduce the carcinogenic effect of ingested nitrate.

Polymorphisms on cytochrome p450 enzymes, like CYP2E1, may also modify carcinogenicity of nitrate and its derivatives. This

enzyme is involved on the bioactivation of some NOCs (nitrosamines), a relevant step preceding genotoxic effects. Polymorphisms on genes coding this enzyme are a susceptibility factor for CRC (Jiang et al. 2013; Le Marchand et al. 2002) and may interplay with nitrate exposure on carcinogenic effects. Gut microbiota is another individual factor that may modify the potential carcinogenic effect of ingested nitrate (Azcárate-Peril et al. 2011), since commensal bacteria is involved in the synthesis of NOCs. This is an open research field for experimental and epidemiological studies on nitrate exposure and cancer outcomes.

Further research is also needed on the interplay of THMs and nitrate associated with cancer risk. Although this was explored in this thesis, more detailed analyses are needed, given the heterogeneous effects of different THM subtypes on cancer risk. A recent analysis of CRC risk and THM levels in drinking water, found inverse associations for chloroform, but positive associations for brominated THM levels (Villanueva et al. Unpublished data). Therefore, future studies on nitrate and THMs in drinking water should evaluate the specific effects of the THMs subtypes on cancer risk.

Finally, the MCC-Spain study offers the opportunity to evaluate the association of nitrate exposure and other cancer outcomes. Gastric cancer is of particular interest given the geographical pattern observed in Spain (highest incidence in traditional agricultural areas). Potential interactions with infectious pathogens (*Helicobacter pylory*), dietary covariates and use of NSAIDs and other drugs may be also evaluated for this prevalent tumor.

7

Conclusions

1. Average current and long-term nitrate levels in residential drinking water were below the European regulatory limit (50 mg/L of NO_3^-) in 10 Spanish and 2 Italian regions. Average levels of waterborne ingested nitrate were lower than levels reported in other countries.

2. Average levels of dietary ingested nitrate were below the current acceptable daily intake (3.7 mg/kg of body weight), and were similar to other western countries. Around 80% of the intake was provided by vegetable sources.

3. Increased risk of bladder cancer was observed only among subjects with long exposure (>20 years) to highest nitrate levels in municipal drinking water (>9.5 mg/L of NO_3^-). Inverse associations were suggested with waterborne ingestion levels, probably confounded by the protective effect of water intake.

4. Increased risk of breast cancer was observed among subgroups of postmenopausal women with high waterborne nitrate and high meat intake. Dietary nitrate was not associated with breast cancer risk, overall. However, positive associations were suggested with nitrate intake from animal sources.

5. Positive associations were suggested for nitrate exposure through drinking water and colorectal cancer risk, particularly among men compared to women. The results were heterogeneous among regions, and statistical significance was affected by the method of statistical analysis applied. Contradictory effects were observed for dietary nitrate intake from animal versus vegetable sources. Only dietary nitrate from animal sources was positively associated with rectal cancer risk.

6. Interactions with endogenous nitrosation factors were not statistically significant for the tumors evaluated. Although, the intake of meat strengthened the associations of waterborne ingested nitrate with breast and colorectal cancer risk.

Appendix

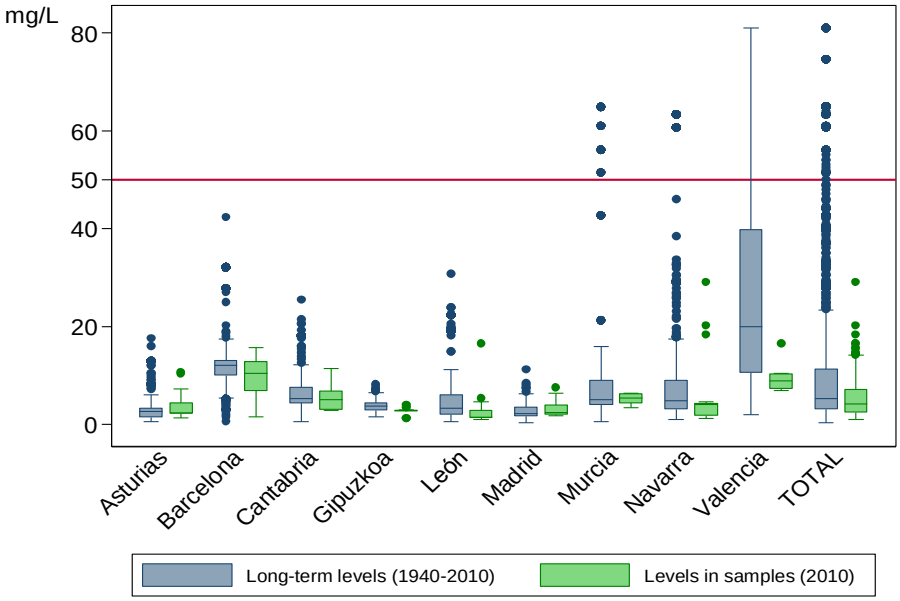
"Doing is being.
To *have* done´s not enough;
To stuff yourself with doing- that´s the game"

(*Ray Bradbury*)

Appendix 1

Nitrate levels (mg/L of NO_3^-) in municipal drinking water

Long-term levels (1940-2010) including measurements and estimates, compared to levels in tap water samples collected in 2010, among study areas



Reference line at 50 mg/L: current regulatory limit in Europe.
Levels >100 mg/L (n=5) were not included in this graphic.

Appendix 2

Nitrate contents (mg/100g) assigned to food items from food frequency questionnaires (FFQs) to calculate dietary nitrate intake

English name	Items listed in the FFQ (MCC-Spain study)[a]	Nitrate content mg/100g portion	Data source
Vegetable sources-Vegetables			
1. Asparagus	Espárragos	20.90	EFSA 2008
2. Beetroot	Otros vegetales (especificar)	185.20	EFSA 2008
3. Belgian endive	Vegetales de hoja verde, escarola, endivias	146.50	EFSA 2008
4. Cucumber	Pepinos	18.50	EFSA 2008
5. French beans	Judías verdes	75.60	EFSA 2008
6. Kale	Col, coliflor, brócoli, coles de Bruselas, repollo	53.70	EFSA 2008
7. Mixed lettuce	Lechuga	206.20	EFSA 2008
8. Mushroom	Champiñones o setas	6.10	EFSA 2008
9. Peas	Guisantes	3.00	EFSA 2008
10. Potato	Patatas cocidas, asadas o puré	16.80	EFSA 2008
11. Pumpkin	Calabaza en temporada	89.40	EFSA 2008
12. Radish	Rábanos	96.70	EFSA 2008
13. Spinach- Silver beet (chard)	Espinacas, acelgas, berros	137.50	EFSA 2008
14. Tomatoes	Tomate crudo	4.30	EFSA 2008
15. Carrots	Zanahoria	16.70	Griesenbeck 2009
16. Squash	Berenjenas, calabacines	42.30	Griesenbeck 2009
17. Yams sweet potato	Boniato en temporada	4.60	Griesenbeck 2009
18. Artichoke	Alcachofa en temporada	1.60	Jakszyn 2004
19. Onion	Cebolla	4.80	Jakszyn 2004
20. Pepper (others)	Pimientos verdes	16.50	Jakszyn 2004
21. Pepper (red)	Pimientos rojos	16.50	Jakszyn 2004

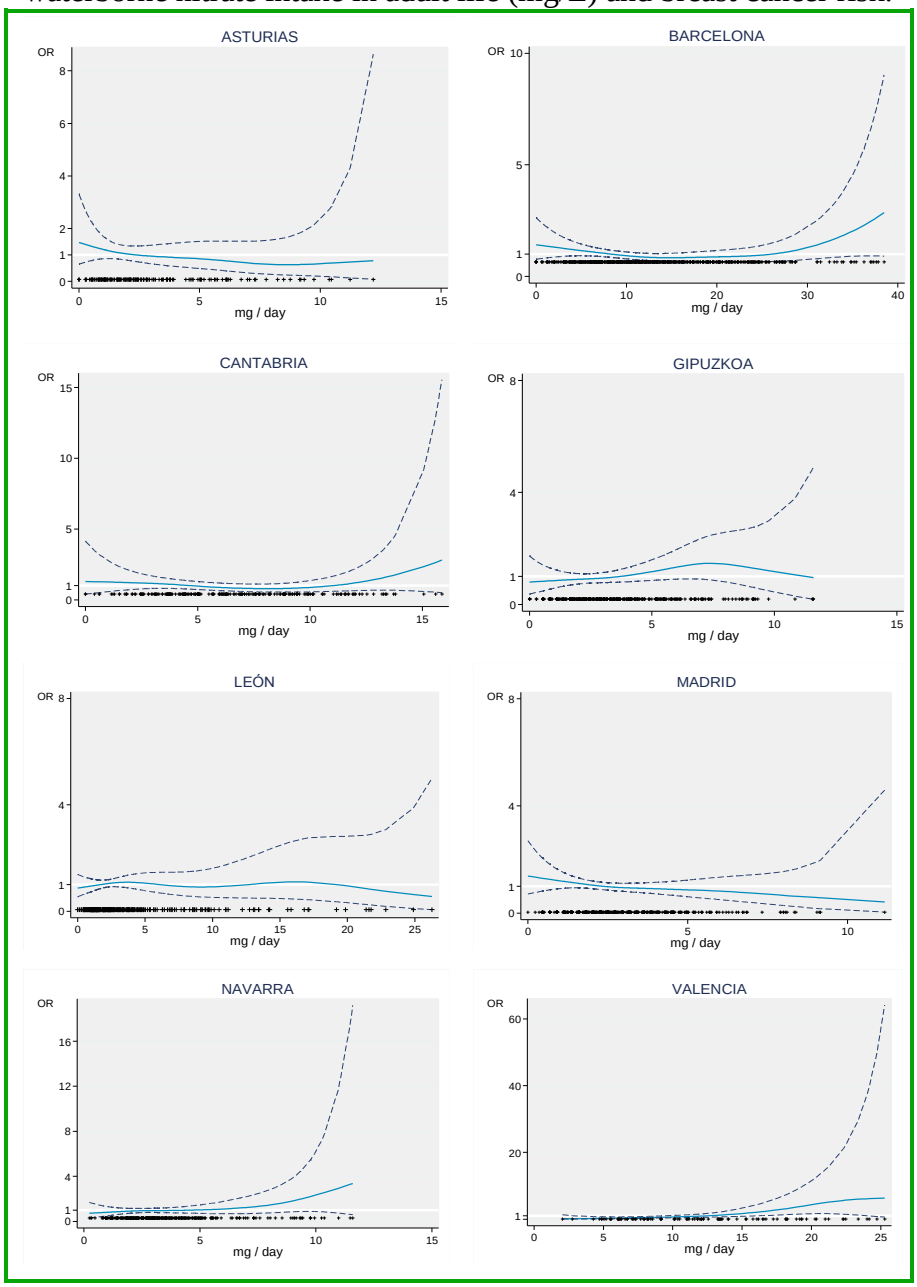
Vegetable sources-fruits				
1.	Apple	Manzana	1.04	Griesenbeck 2009
2.	Avocado	Aguacate en temporada	2.63	Griesenbeck 2009
3.	Banana	Plátano	2.00	Griesenbeck 2009
4.	Cantaloupe	Sandía, melón en temporada	9.48	Griesenbeck 2009
5.	Cherry	Cerezas o picotas en temporada	2.62 [b]	Griesenbeck 2009
6.	Fig	Higos frescos en temporada	2.62 [b]	Griesenbeck 2009
7.	Grape (Other fruits)	Uva	2.62 [b]	Griesenbeck 2009
8.	Kiwi	Kiwi	2.62 [b]	Griesenbeck 2009
9.	Mango, papaya	Mango o papaya en temporada	2.62 [b]	Griesenbeck 2009
10.	Orange	naranja, pomelo, mandarinas	2.00	Griesenbeck 2009
11.	Peaches, apricots	Melocotón, nectarina, en temporada	0.64	Griesenbeck 2009
12.	Plums	Ciruela en temporada	2.62 [b]	Griesenbeck 2009
13.	Strawberry	Fresas o fresones en temporada	2.62 [b]	Griesenbeck 2009
Animal sources				
1.	Chicken	Pollo	0.66	Griesenbeck 2009
2.	Eggs	Huevos fritos, duros, tortilla	0.53	Griesenbeck 2009
3.	Lamb	Carne de cordero o cabrito	5.80	Griesenbeck 2009
4.	Liver	Hígado de ternera, cerdo o pollo	12.56	Griesenbeck 2009
5.	Pork	Carne de cerdo	5.86	Griesenbeck 2009
6.	Rabbit	Conejo o liebre	5.80	Griesenbeck 2009
7.	Skim or low fat milk	Leche desnatada o semi-desnatada	0.35	Griesenbeck 2009
8.	Yogurt	Yogurt descremado	0.14	Griesenbeck 2009
9.	Hot dogs/sausages	Frankfurt y similares	37.0	Jakszyn 2004 +Griesenbeck 2009
10.	Bacon	Bacon, tocino o panceta	9.40	Jakszyn 2004
11.	Beef	Carne de ternera, buey, vaca o res	6.12	Jakszyn 2004
12.	Cheese (cured)	Queso curado o semicurado	3.20	Jakszyn 2004

13.	Cured ham	Jamón salado, serrano o país	25.00	Jakszyn 2004
14.	Foie gras (pate liver)	Patés foie gras	18.3	Jakszyn 2004
15.	Fresh cheese	Queso blanco o fresco	1.61	Jakszyn 2004
16.	Ham	Jamón dulce, York o cocido	1.90	Jakszyn 2004
17.	Spanish sausages(fuet)	Fuet, salchichón o chorizo curado	3.30	Jakszyn 2004
Others				
1.	Pasta	Pasta sin salsa	1.64	Griesenbeck 2009
2.	Rice	Arroz	1.64	Griesenbeck 2009
3.	White bread	Pan Blanco	2.50	Jakszyn 2004
4.	Beer	Cerveza con alcohol	1.70	Jakszyn 2004

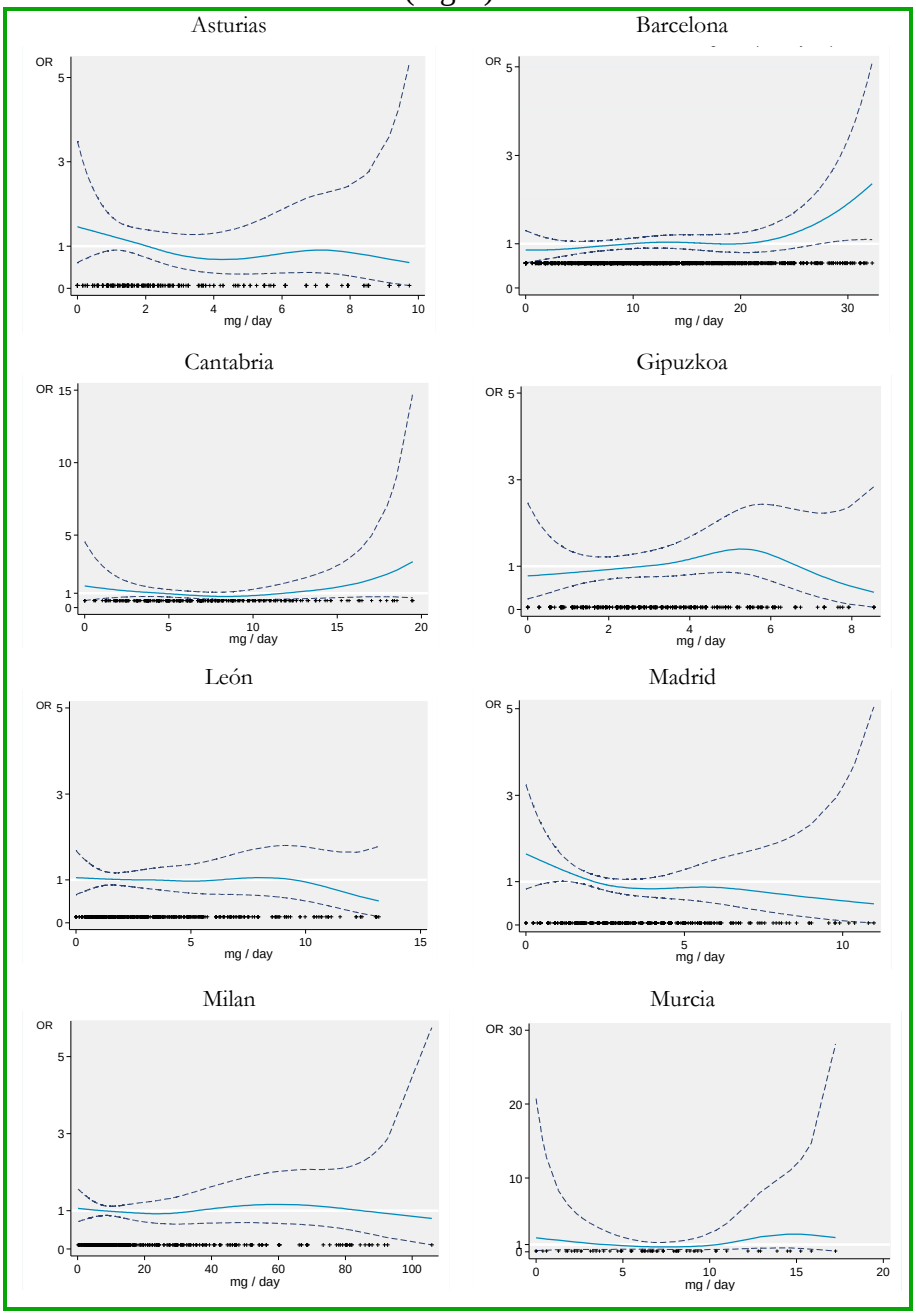
[a] Food items listed in the FFQ from Italy were similar. [b] Levels reported as "Other fruits" in Griesenbeck 2009 were assigned to these items.

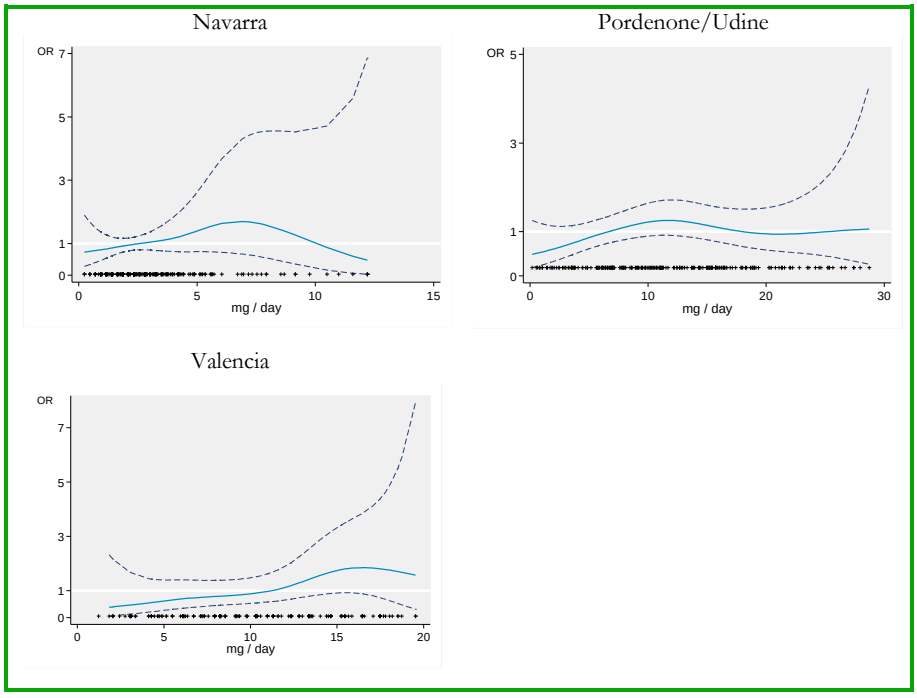
Appendix 3

Appendix 3.1 Generalized additive models (GAMs) by study area (paper III) Exposure-response relationship between waterborne nitrate intake in adult life (mg/L) and breast cancer risk.



Appendix 3.2 Generalized additive models (GAMs) by study area (paper IV) Exposure-response relationship between waterborne nitrate intake (mg/L) and colorectal cancer risk.





Waterborne ingested levels >p99 in each area were excluded from the GAMs
GAMs in Appendix 1.1 were adjusted for: age, education, body mass index, family history of breast cancer and energy intake.
GAMs in Appendix 1.2 were adjusted for sex, age, education, body mass index, physical activity, non-steroidal anti-inflammatories use, family history of colorectal cancer, energy and alcohol intake.

Appendix 4

Meta-smoothing analysis

This analysis was conducted to evaluate the exposure-response relation between nitrate levels and colorectal cancer (CRC) risk among our study areas (9 areas from Spain and 2 from Italy). The meta-smoothing enabled us to obtain one single exposure-response curve, combining the heterogeneous curves observed among the study areas. This approach was developed for air pollution studies (Schwartz and Zanobetti 2000; Stafoggia et al. 2013) and has not been applied in studies on water contaminants. The analysis was conducted in R version 3.1.0 (R Development Core Team 2014, **http://R-project.org**). The results of this analysis were not included in the final manuscript (paper IV), since were similar to results of generalized additive models (GAMs) presented in the manuscript.

a) Steps for the analysis

1. First we tested non-linearity of the dose-response relation between residential (or waterborne ingested) nitrate levels and CRC risk in each study area, using generalized additive models (GAMs) (see an example in the next page). For each study area we fitted logistic regression models (when linear association was observed) or GAM models. All models included the same confounders: age, sex, education, body mass index, family history of CRC and physical activity.

2. We defined several cutoffs for waterborne ingested levels (0, 5, 10, 15, 20, 25, 30, 35, 40, and 45 mg/day) and for residential nitrate levels (2, 4, 6, 8, 10, 12, 15, 20, 25 and 30 mg/L). Cut-offs were considered valid for each area when the values of the cut-offs were observed among the nitrate levels of the area. Therefore, two or more areas were represented in each cut-off.

3. For predictions of CRC risk: we fixed the value of the confounders. This value was the mean for continuous variables (body mass index) and the category of reference for categorical variables (e.g. Men for sex). Then, we obtained a predicted

value (logodds and standard errors) for each cut-off of the exposure and each study area.

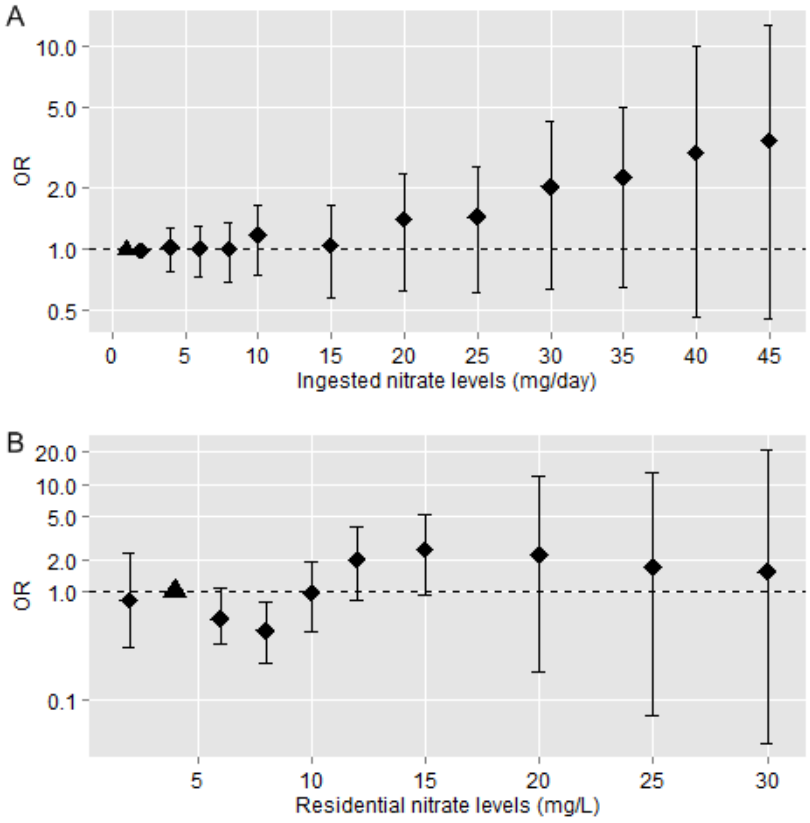
4. We pooled the area specific results using a random-effects meta-analysis, and we obtained global log-odds for each cutoff.

5. Confidence intervals of each global log-odd were obtained using bootstrapping methods with 1000 iterations (Normal approximation method). The boot package was used for bootstrapping.

6. Results are expressed as Odd ratios and 95% confidence intervals (see graphic below).

b) Results of the meta-smoothing analysis*

Exposure-response between colorectal cancer risk (Odds ratios OR) and (A) waterborne ingested levels and (B) average residential nitrate levels. Adjusted for study area, sex, age, education, physical activity, body mass index, use of non-steroidal anti-inflammatory drugs, family history of colorectal cancer, energy and ethanol intake. ▲Reference levels: 1mg/day (panel A) and 4mg/L (panel B). Confidence intervals were computed by bootstrap (normal approximation method) with 1000 iterations.



*This analysis was conducted among population with nitrate residential levels available for >70% of the exposure period in adult life (age 18 to 2 years before recruitment n=5131)

Appendix 5

Sensitivity analyses with mixed models (paper IV)

Colorectal cancer risk (Odds ratios and 95% confidence intervals) and waterborne nitrate exposure. Results of mixed models with area as random effect, excluding areas with >10% of cases (n=5399)

			Logistic regression	**Mixed models with area as random effect**				
Exposure in the last 30 years before recruitment*	**Cases**	**Controls**	**Main results** (all areas) **OR (95%CI)**	All areas **OR (95%CI)**	Excluding: Milan **OR (95%CI)**	Barcelona **OR (95%CI)**	León **OR (95%CI)**	Madrid **OR (95%CI)**
Mean waterborne ingestion								
≤5 mg/day	778	1899	1[a]	1[a]	1[a]	1[a]	1[a]	1[a]
>5-10 mg/day	447	803	1.14 (0.96, 1.36)	1.17 (1.00, 1.40)	1.13 (0.93, 1.38)	1.12 (0.91, 1.37)	1.25 (1.04, 1.51)	1.18 (0.99, 1.41)
>10 mg/day	644	828	1.45 (1.21, 1.74)	1.52 (1.27, 1.83)	1.86 (1.52, 2.29)	1.44 (1.14, 1.82	1.62 (1.33, 1.97)	1.55 (1.29, 1.87)
Mean residential levels								
≤5mg/L	699	1788	1[b]	1[b]	1[b]	1[b]	1[b]	1[b]
>5<10 mg/L	325	796	1.25 (0.89, 1.78)	1.12 (0.82, 1.53)	1.16 (0.86, 1.58)	1.41 (1.04, 1.91)	1.20 (0.84, 1.73)	1.11 (0.81, 1.54)
≥10 mg/L	845	946	4.46 (3.01, 6.62)	3.68 (2.59, 5.23)	3.91 (2.76, 5.54)	2.36 (1.62, 3.45)	3.84 (2.58, 5.71)	3.70 2.55, 5.36)

*The main exposure period comprised 30 years before recruitment except the last 2 years of exposure. [a] Adjusted for: area, sex, age, education, smoking, intake of energy, vitamin E, vitamin C, red meat, body mass index, physical activity, non-steroidal anti-inflammatories use, family history of colorectal cancer. [b] Adjusted for: area, sex, age, education, body mass index, physical activity, non-steroidal anti-inflammatories use, family history of colorectal cancer, intake of energy, vitamin E and red meat.

Appendix 6

Residential information, nitrate levels in municipal water and years consuming different types of water in adult life

Appendix 6.1 Among women analyzed in the paper III

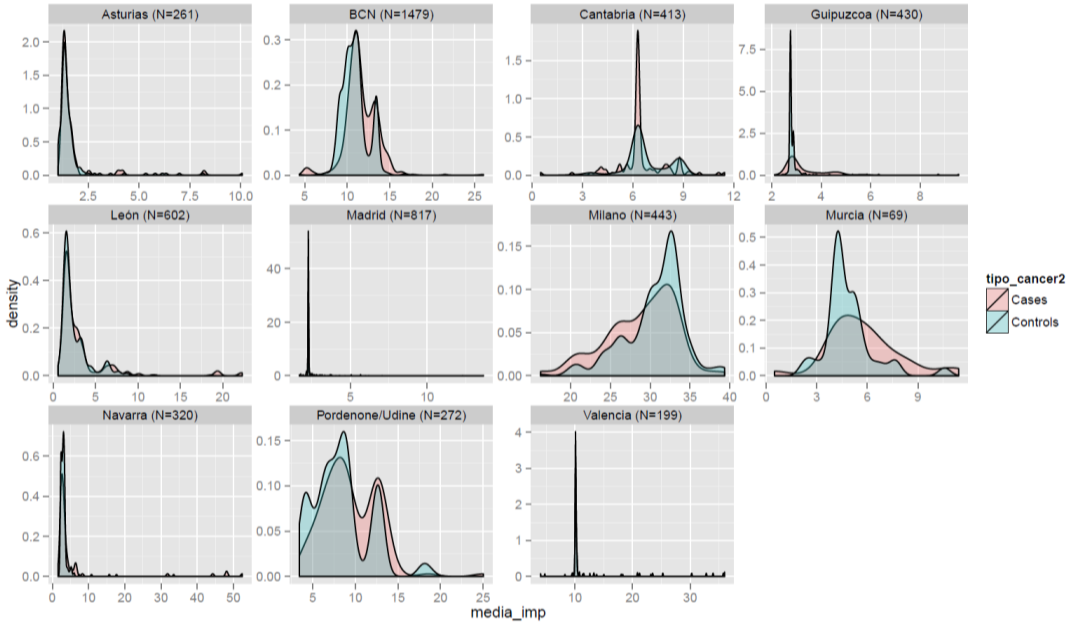
Study area	Women N	Residential information (years)	Nitrate levels available (years)		Type of water consumed in the residences (years)		
			Measurements +estimations	Only measurements	Municipal	Bottled	Well/other
		Mean(SD)	Mean(SD)	Mean(SD)	Mean(SD)	Mean(SD)	Mean(SD)
Asturias	169	38.3 (12.7)	36.8 (12.1)	12,2 (2.4)	29.6 (15.9)	6.2 (11.8)	2.4 (5.9)
Barcelona	598	41.6 (12.3)	40.1 (12.1)	4,04 (1.2)	24.3 (20.4)	15,9 (16.9)	1.1 (4.2)
Cantabria	237	35,6 (12.0)	34.4 (11.8)	4,41 (1.4)	28.3 (16.4)	5.9 (10.6)	1.4 (5.5)
Guipuzcoa	417	39.2 (12.1)	37.8 (12.0)	13,4 (1.7)	32.4 (16.3)	5.4 (12.0)	0.9 (3.8)
León	323	40.3 (13.2)	38.5 (12.8)	3,42 (1.4)	28.8 (18.2)	3.5 (9.9)	7.9 (13.8)
Madrid	585	36.9 (13.0)	35.1 (12.4)	3,83 (1.0)	34.7 (13.4)	1.4 (5.8)	0.7 (2.8)
Navarra	329	39.3 (13.4)	37.7 (12.9)	5,01 (1.9)	36.0 (16.2)	3.1 (9.8)	0.2 (1.4)
Valencia	107	40.6 (12.0)	39.1 (11.7)	6,16 (1.6)	21.6 (19.9)	16.3 (17.2)	2.3 (7.6)
Total	2765	39.1 (12.8)	37.5 (12.4)	6,06 (4.0)	30.2 (17.6)	6.9 (13.2)	1.8 (6.5)

Appendix 6.1 Among population analyzed in the paper IV

Study area	Population	Residential information	Nitrale levels available (years)		Type of water consumed in the residences (years)		
	N	(years)	Measurements +estimations	Only measurements	Municipal	Bottled	Well/other
		Mean(SD)	Mean(SD)	Mean(SD)	Mean(SD)	Mean(SD)	Mean(SD)
Asturias	261	44.5 (12.2)	41.5 (11.8)	12.4 (2.3)	31.7 (17.6)	8.8 (14.5)	3.5 (6.9)
BCN	1539	47.5 (10.0)	43.9 (10.0)	4.0 (1.7)	29.1 (19.9)	15.6 (17.9)	2.0 (4.8)
Cantabria	398	44.0 (10.6)	40.5 (10.7)	4.3 (1.1)	34.5 (17.3)	7.7 (14.3)	1.1 (3.7)
Guipuzcoa	440	43.7 (11.4)	41.1 (11.3)	13.4 (1.5)	35.6 (16.8)	6.2 (13.3)	0.9 (3.5)
León	595	47.6 (11.7)	43.3 (12.0)	3.3 (1.4)	30.9 (20.4)	5.0 (12.4)	10.9 (16.2)
Madrid	864	44.2 (11.7)	40.5 (10.9)	4.0 (0.8)	41.1 (12.6)	1.6 (6.5)	1.1 (3.4)
Milano	454	46.9 (10.0)	44.3 (10.3)	7.3 (1.5)	19.4 (20.6)	26.9 (19.9)	0.5 (2.0)
Murcia	64	44.2 (11.8)	41.8 (11.8)	5.9 (3.2)	23.9 (18.1)	15.3 (17.5)	4.5 (9.7)
Navarra	328	46.2 (11.4)	42.3 (11.2)	4.6 (1.4)	42.1 (14.8)	2.8 (9.7)	0.6 (3.0)
Pordenone/Udine	249	45.1 (9.0)	40.8 (9.9)	9.3 (3.7)	31.6 (19.5)	9.8 (14.8)	3.6 (7.4)
Valencia	207	46.6 (10.2)	43.7 (10.4)	6.2 (1.4)	26.4 (20.2)	18.1 (18.4)	1.5 (4.9)
Total	**5399**	**45.9 (10.9)**	**42.4 (10.8)**	**5.8 (3.6)**	**32.2 (19.2)**	**10.5 (16.6)**	**0.8 (4.2)**

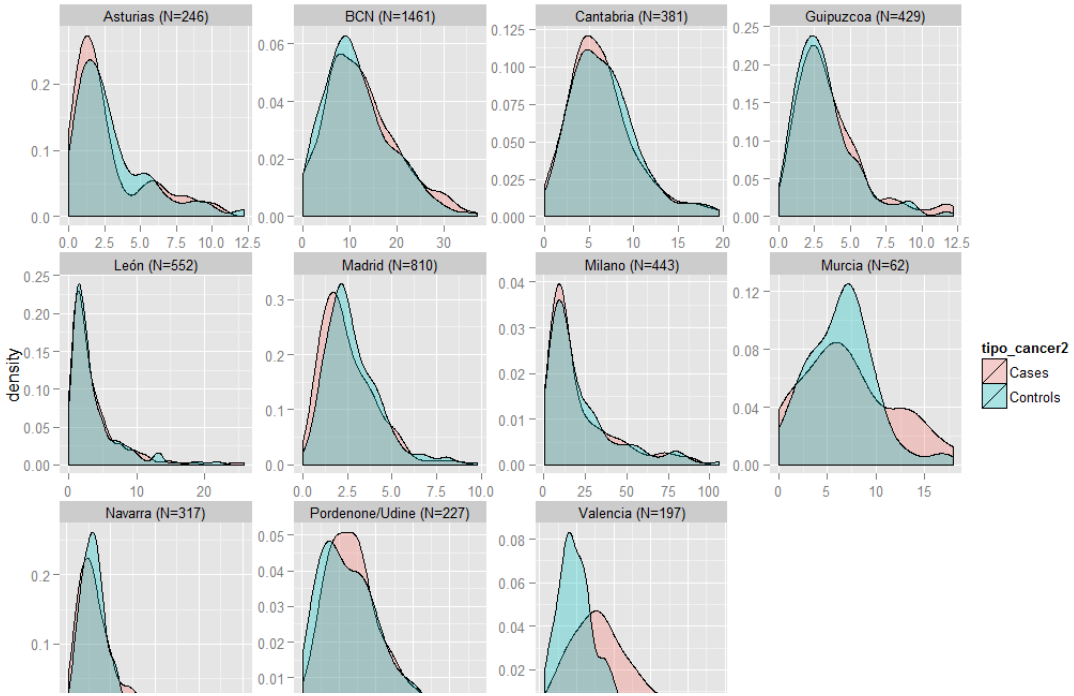
Appendix 7

Appendix 7.1 Distribution (density) of cases of colorectal cancer and controls according to average residential nitrate levels (mg/L of NO3-) in adult life*



*This analysis included population with nitrate levels covering >70% of adult life. This group was analyzed in preliminary analyses of the paper IV, but results are not shown in the final manuscript.

Appendix 7.2 Distribution (density) of cases of colorectal cancer and controls according to average waterborne ingested nitrate levels (mg/day) in adult life*



*This group of population was included in preliminary analyses of the paper IV, but results are not shown in the final manuscript.

Appendix 8

Analyses on waterborne intake adjusted for dietary intake of nitrate. Papers III and IV

Appendix 8.1 Breast cancer risk (OR and 95%CI) associated with waterborne ingested nitrate in adult life adjusted for dietary ingested nitrate

Mean waterborne ingested nitrate				Additionally adjusted for nitrate intake from:		
				Total diet	Animal sources	Vegetable sources
	Cases	Controls	OR [a] (95%CI)	OR [a] (95%CI)	OR [a] (95%CI)	OR [a] (95%CI)
Postmenopausal women (n=2086)						
<2.7mg/day*	300	391	1	1	1	1
≥2.7-6.5 mg/day	304	392	1.10 (0.87, 1.38)	1.19 (0.93, 1.51)	1.17 (0.92, 1.49)	1.19 (0.93, 1.51)
>6.5 mg/day	309	390	1.28 (0.94, 1.72)	1.34 (0.97, 1.84)	1.30 (0.94, 1.79)	1.33 (0.97, 1.84)
Premenopausal women (n=679)						
<2.3 mg/day*	112	116	1	1	1	1
≥2.3-4.7 mg/day	98	116	0.85 (0.57, 1.26)	0.87 (0.57, 1.33)	0.87 (0.57, 1.33)	0.87 (0.57, 1.33)
>4.7 mg/day	122	115	1.00 (0.64, 1.58)	1.09 (0.67, 1.80)	1.13 (0.69, 1.86)	1.10 (0.67, 1.80)

[a] Adjusted for: area, age, education, body mass index, family history of breast cancer, use of non-steroidal anti-inflammatories and energy intake.

Appendix 8.2 Colorectal cancer risk (OR and 95%CI) associated with waterborne ingested nitrate during the main exposure period* adjusted for dietary ingested nitrate

Overall population (n=5399)				Additionally adjusted for nitrate intake from:		
				Total diet	Animal sources	Vegetable sources
Mean waterborne ingested nitrate	Cases	Controls	OR [a] (95%CI)	OR [a] (95%CI)	OR [a] (95%CI)	OR [a] (95%CI)
≤5 mg/day	778	1899	1	1	1	1
>5-10 mg/day	447	803	1.14 (0.96, 1.36)	1.06 (0.88, 1.28)	1.06 (0.88, 1.27)	1.06 (0.89, 1.28)
>10 mg/day	644	828	1.45 (1.21, 1.74)	1.32 (1.09, 1.60)	1.30 (1.08, 1.58)	1.32 (1.09, 1.60)

*The main exposure period comprise 30 years before recruitment excluding the last 2 years of exposure. [a] Adjusted for: area, sex, age, education, body mass index, physical activity, use of non-steroidal anti-inflammatories, family history of colorectal cancer, intake of energy, vitamin E and alcohol.

Appendix 9

Additional analyses on colorectal cancer risk associated with nitrate and trihalomethane exposure (paper IV)

Appendix 9.1
Spearman correlations coefficients (*r*) for trihalomethane and nitrate exposure levels in adult life (N=5009)*

		THM exposure			Nitrate exposure			
		Residential levels			Residential levels		Waterborne ingested	
		TTHMs	chcl3	Tbrom	(A)	(B)	(A)	(B)
THM exposure	Total THMs	1.00						
	Chloroform (chcl3)	0.65	1.00					
	Total brominated (Tbrom)	0.80	0.20	1.00				
Nitrate exposure	Residential levels (A)	0.11	-0.42	0.28	1.00			
	Residential levels (B)	0.11	-0.44	0.29	0.98	1.00		
	Waterborne ingested (A)	0.12	-0.30	0.23	0.68	0.68	1.00	
	Waterborne ingested (B)	0.14	-0.28	0.25	0.64	0.65	0.98	1.00

*Only subjects with trihalomethane levels available for >70% of the adult life exposure period were analyzed. THM: trihalomethane. TTHMs: total trihalomethane levels. Tbrom: total brominated trihalomethane levels. (A): nitrate exposure in adult life. (B): nitrate exposure for 30 years before recruitment (excluding the last 2 years of exposure).

Appendix 9.2

Association of colorectal cancer risk and residential nitrate levels (mg/L) adjusted for trihalomethane residential levels (µg/L). Exposure in adult life (from age 18 to 2 years before recruitment)

Residential nitrate levels	Cases	Controls	OR[a]	95%CI	Adjusted for TTHMs[b,c] OR	95%CI	Chloroform [c] OR	95%CI	Total brominated [c] OR	95%CI
All population (n=5006)*										
≤5mg/L	602	1664	1		1		1		1	
>5<10 mg/L	261	608	1.40	(0.98, 2,01)	1.48	(1.03, 2.13)	1.15	(0.79, 1.66)	1.45	(1.01, 2.09)
≥10 mg/L	817	1054	3.87	(2.55, 5.87)	3.70	(2.44, 5.61)	2.80	(1.82, 4.31)	4.00	(2.64, 6.07)
p for interaction					*0.001*		*<0.001*		*0.120*	
Men (n=2781)										
≤5mg/L	384	718	1		1		1		1	
>5<10 mg/L	170	394	1.24	(0.77, 1.99)	1.32	(0.82, 2.12)	1.00	(0.62, 1.63)	1.27	(0.79, 2.05)
≥10 mg/L	511	604	4.46	(2.58, 7.71)	4.18	(2.41, 7.22)	3.06	(1.73, 5.40)	4.57	(2.64, 7.92)
p for interaction					*0.026*		*<0.001*		*0.230*	
Women (n=2225)										
≤5mg/L	218	946	1		1		1		1	
>5<10 mg/L	91	214	1.65	(0.92, 2.97)	1.72	(0.95, 3.10)	1.37	(0.75, 2.52)	1.74	(0.97, 3.11)
≥10 mg/L	306	450	2.48	(1.26, 4.88)	2.45	(1.24, 4.82)	2.02	(1.01, 4.06)	2.61	(1.33, 5.13)
p for interaction					*0.088*		*0.0001*		*0.220*	

*Only subjects with trihalomethane levels available for >70% of the adult life exposure period were analyzed. [a] Adjusted for area, age, sex, education, use of non-steroidal anti-inflammatories, smoking, physical activity, family history of colorectal cancer. [b] TTHMs: Total trihalomethane levels. [c] Levels of trihalomethanes were included in the adjustment as continuous variables.

Appendix 10

About the Author

Nadia Carminia Espejo Herrera was born in La Paz- Bolivia in 1983. She received her degree in Medicine in 2007 at the University "Mayor de San Andrés" in La Paz. In 2009, she obtained a Carolina Foundation´s scholarship and was enrolled in the Master of Public Health at the Pompeu Fabra University and Autonoma University of Barcelona (Spain). She joined the Centre for Research in Environmental Epidemiology (CREAL) in 2010, and conducted this thesis between 2011 and 2015. Research activities developed by the author during her PhD studies are summarized below.

La Paz-Bolivia (2011)

Summary of PhD activities

1. **Project defense (April 2011)**
2. **First year after project defense (2011-2012)**

Data collection and statistical analyses

- Contact with local governments and water suppliers to collect additional data on water source, treatment methods, contaminant levels, etc.
- Compilation of data bases for modeling historical nitrate levels and evaluation of long-term exposure.
- Sampling of bottled water brands (Barcelona).
- Description of water consumption habits in population-based controls of 10 Spanish study areas.

Meetings and presentations

23rd International ISEE (International Society of Environmental Epidemiology) Conference. September 13th to 16th 2011 (Barcelona, Spain). Poster submitted: "Water consumption habits and levels of nitrate and trace metals in municipal water multicase-control study (MCC-Spain)".

Training activities

Course of "Técnicas actorales para la comunicación científica" (Intervals program PRBB).

3. **Second year (2012-2013)**

Data collection and analyses

- Modeling of historical nitrate level based on the environmental information available in municipalities from Barcelona, Madrid, Navarra and Gipuzkoa.
- Description of residential history and water consumption habits in Barcelona, Gipuzkoa, Madrid and Navarra.

Publications

Paper I "Nitrate and metals in municipal and bottled water in Spain". Admitted for publication in Gaceta Sanitaria

Meetings and presentations

- 2nd International Congress on Environmental Health (ICEH). May 29th –June 2nd, 2012. Lisbon- Portugal. Oral communication: "Assessment of long term exposure to nitrate in

drinking water and methods to address exposure misclassification".

- Annual MCC- Spain study meeting. October 8th- 9th, 2012. Granada- Spain. Communication of advances in long term exposure assessment to nitrate in study areas and methodological issues.
- Meetings with of Water research group of MCC- Spain study.
- "Current analyses in the Water working group". Presentation in CREAL´s annual scientific retreat.

Training activities

"Scientific writing". Basic course on scientific writing organized by Intervals. PRBB.

4. Third year (2013-2014)

Data collection and analyses

- Well water sampling organized and performed in municipalities from León (September 2013).
- Evaluation of long-term exposure to nitrate in municipalities from nine Spanish study areas and municipalities from Milan, Pordenone and Udine, in coordination with researchers from the Institute Mario Negri (Milan- Italy).
- Evaluation of nitrate in drinking water and bladder cancer in Spain completed (paper II).
- Evaluation of nitrate in drinking water and colorectal cancer started (paper III)

Meetings and presentations

- Iberoamerican Congress on Epidemiology and Public Health. Annual meeting of the Spanish Society of Epidemiology (SEE) September 4th –6th, 2013. Granada- Spain. Oral communication: "Nitrate in drinking water and risk of bladder cancer in Spain".
- "Nitrato y metales en agua de consumo". Teaching activity at the formative courses for environmental workers in local government of Barcelona (Diputació de Barcelona).
- Participation in the MCC- Spain study, presentation of results.
- "Nitrate in drinking water and bladder cancer-update". Presentation in CREAL´s annual scientific retreat.

Training activities

- "Write it clearly". Intermediate course on scientific writing organized by Intervals. PRBB (May-June, 2013).
- Courses of programming, elaboration of dynamic tables and advanced graphics in Excel and other courses organized for CREAL members.

Peer reviewer

For Environmental Engineering and Management Journal. Original articles.

Publications

Paper II "Nitrate in drinking water and bladder cancer risk in Spain" (Espejo-Herrera N, Cantor KP, Malats N, Silverman DT, Tardón A, García-Closas R, Serra C, Kogevinas M, Villanueva CM) Submitted to Environmental Research.

5. Fourth year (2014-2015)

Data analyses

- Evaluation of breast cancer risk in eight Spanish regions.
- Evaluation of colorectal cancer risk in nine Spanish and two Italian regions.
- Proposal for new analyses: Nitrate exposure during pregnancy, pregnancy outcomes and thyroid function in the framework of the INMA project.

Meetings and presentations

- "Nitrate in drinking water and bladder cancer risk in Spain" presented in a scientific session of the Working group on cancer research in CREAL.
- Annual meeting of the International Society of Environmental Epidemiology (ISEE). August 24th- 28th, 2014. Seattle- United States. Oral communication "Long-term exposure to nitrate in drinking water and colorectal cancer risk (MCC-Spain study).
- International Society of Environmental Epidemiology (ISEE) for young researchers as an oral communication (Barcelona, October 20th and 21st 2014).
- "Nitrate exposure and colorectal cancer risk". Presented in a CREAL scientific session.

Training activities

- Sessions of the annual meeting of the International Society of Environmental Epidemiology (ISEE). August 24th- 28th, 2014. Seattle- United States
- Advanced course of scientific writing "Writing for the reader" of the Intervals program in the PRBB.
- On- line course of Global Health (Coursera) on the University of de Geneve (8 weeks of follow-up).

Peer reviewer

- For Gaceta Sanitaria. Original article (1).
- Reviewer in the II Iberoamerican Congress on Epidemiology and Public Health. Annual meeting of the Spanish Society of Epidemiology (SEE) Santiago de Compostela- Spain (10 abstracts).

Publications

- Paper II "Nitrate in drinking water and bladder cancer risk in Spain" Espejo-Herrera N, Cantor KP, Malats N, Silverman DT, Tardón A, García-Closas R, Serra C, Kogevinas M, Villanueva CM. Published in Environmental Research.
- Paper III and paper IV. Reviewed by co-authors and submitted for publication.

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