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Colorectal Cancer and Long-Term Exposure to Trihalomethanes in Drinking Water: A Multicenter Case-Control Study in Spain and Italy

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Abstract

Background: Evidence on the association between colorectal cancer and exposure to disinfection by-products in drinking water is inconsistent.

Objectives: We assessed long-term exposure to trihalomethanes (THMs), the most prevalent group of chlorination by-products, to evaluate the association with colorectal cancer.

Methods: A multicentre case-control study was conducted in Spain and Italy in 2008-2013. Hospital-based incident cases, population-based (Spain) and hospital-based (Italy) controls were interviewed to ascertain residential histories, water type consumed in each residence, frequency and duration of showering/ bathing, and major recognized risk factors for colorectal cancer. We estimated adjusted odds ratios (OR) for colorectal cancer in association with quartiles of estimated average lifetime THM concentrations in each participant's residential tap water (μg/L, from age 18 to two years before the interview) and estimated average lifetime THM ingestion from drinking residential tap water (μg/day).

Results: Subjects analyzed were 2047 cases and 3718 controls. Median values (ranges) for average lifetime residential tap water concentrations of total THMs, chloroform, and brominated THMs were 30 (0-174), 17 (0-63), and 9 (0–145) μg/L, respectively. Total THM concentration in residential tap water was not associated with colorectal cancer (OR=0.92, 95%CI: 0.66-1.28 for highest vs. lowest quartile), but chloroform concentrations were inversely associated (OR=0.31, 95%CI: 0.24-0.41 for highest vs. lowest quartile). Brominated THMs concentrations showed a positive association among men at the highest vs. lowest quartile (OR=1.43, 95%CI: 0.83-2.46). Patterns of associations were similar for estimated average THM ingestion through residential water consumption.

Conclusions: We did not find clear evidence of an association between detailed estimates of lifetime total THM exposures and colorectal cancer in our large case-control study population. Negative associations with chloroform concentrations and ingestion suggest differences among specific THMs, but these findings need confirmation in other study populations.

INTRODUCTION

Colorectal cancer represents almost 10% of the global cancer incidence, with increasing rates over the last decades (Bosman et al. 2014). Intake of total energy, red and processed meat, and alcohol drinks together with physical inactivity, body fatness, abdominal fatness and adult attained height are established risk factors (Bosman et al. 2014; WCRF et al. 2011). Suggested protective factors include dietary fiber and high fruit and vegetable consumption (WCRF et al. 2011; Bradbury et al. 2014), among others. However, part of the burden of disease remains unexplained by these risk factors. Human and experimental studies suggest that carcinogens in drinking water may be associated with colorectal cancer risk (Rahman et al. 2010).

Disinfection by-products (DBPs) are widespread chemicals in drinking water resulting from disinfection processes. Trihalomethanes (THMs) are among the most prevalent DBPs and are highly volatile and skin permeable, and exposure occurs through inhalation, dermal absorption and ingestion (Ashley et al. 2005; Gordon et al. 2006). The four regulated THMs in drinking water in the USA, EU, and other countries include chloroform, bromodichloromethane, dibromochloromethane, and bromoform, and show different physico-chemical and toxicological properties. Chloroform is highly volatile, while the other 3 (from now on referred as brominated THMs) are more lipophilic and genotoxic (Plewa et al. 2008). Metabolism of DBPs is mediated by enzymes from the cytochrome P450 (CYP) and glutathione S-transferase (GST) families. Polymorphisms in *CYP2E1*, *GSTT1* and *GSTZ1* modified associations between bladder cancer and DBP exposure in a hospital-based case control study (Cantor et al. 2010).

Animal studies suggest an association between DBP exposure and colorectal cancer. Preneoplastic lesions have been produced in the intestines of rodents administered DBPs via drinking water in chronic bioassays (DeAngelo et al. 2002; McDorman et al. 2003). However, some studies have shown chloroform may inhibit gastrointestinal carcinogenecity in rodents (Daniel et al. 1989; Daniel et al. 1991). Human epidemiological evidence is mixed. Case-control (Bove, Jr. et al. 2007; Cragle et al. 1985; Hildesheim et al. 1998; King et al. 2000; Young et al. 1987) and cohort (Doyle et al. 1997; Koivusalo et al. 1997) studies including incident cancer cases of colorectal cancer and quantitative estimates of DBP exposure such as trihalomethanes or related surrogates show contradictory results (see Supplementary Material, Table S1). Three studies reported positive associations with colon cancer (King et al. 2000; Cragle et al. 1985; Doyle et al. 1997) and three studies reported null associations (Hildesheim et al. 1998; Koivusalo et al. 1997; Young et al. 1987). Similarly, two studies reported positive associations with rectal cancer (Bove, Jr. et al. 2007; Hildesheim et al. 1998) and three reported null associations (King et al. 2000; Koivusalo et al. 1997; Doyle et al. 1997). Exposure assessment differed, with five studies evaluating THMs (Bove, Jr. et al. 2007; Doyle et al. 1997; Hildesheim et al. 1998; King et al. 2000; Young et al. 1987) and one each evaluating years living in households with chlorinated water (Cragle et al. 1985), and water mutagenicity (Koivusalo et al. 1997). The inconsistency of human epidemiological evidence might be partly attributable to exposure misclassification including the lack of evaluation of different routes of exposure and uncontrolled confounders.

We examined the association between THM exposure and colorectal cancer in a large multicentre case-control study considering specific THM components and exposure activities involving dermal contact, inhalation and ingestion.

METHODS

Study design and population

Spain. We conducted a multicase case-control study from September 2007 to November 2013 in nine provinces of Spain (MCC-Spain project) including colorectal, prostate, breast, and gastro-esophageal cancer and chronic lymphocytic leukemia cases, and a common pool of population-based controls (Castano-Vinyals et al. 2015). Cases included in the present analysis were incident colorectal cancer patients histologically confirmed (International Classification of Diseases, ICD-10: C18, C19, C20, D01.0, D01.1, D01.2). Cases were interviewed as soon as possible after the diagnosis (median of 58 days) through active searches including periodical visits to the hospital departments (i.e. gastroenterology, oncology, general surgery, radiotherapy and pathology). Minimal losses were produced because of cases dying before being contacted (0.5% of potentially eligible cases). Controls were frequency matched to cases by sex, age (±5 years), and area of residence, ensuring that in each area there was at least one control of the same sex and 5-year interval for each case. Controls were selected from the general population in each study area, identified from the lists of randomly selected family practitioners in primary assistance centers sharing the catchment area of the participating hospital. This procedure ensured the identification of subjects from the general population and the same study base as cases, through the social security records (health coverage is universal in Spain), with the advantage that the telephone contacts by the study personnel on behalf of the family practitioner increased the response rate compared to other procedures (Castano-Vinyals et al. 2011). For each control needed, five potential participants of similar age, sex and hospital catchment area were randomly selected from the general practitioner lists. If contact with the first person of this list was not possible after five tries at different times of the day, or if he/she refused to participate, the following person of the list was approached (Castano-Vinyals et al. 2011). The frequency

matching was done separately for each study area, considering the age and sex distribution of the total number of cases recruited. Thus, the final number of controls in the present analysis is higher than cases because they are matched to different cancer sites.

Italy. We conducted a case-control study of colorectal cancer in three provinces of Italy (Milan, Pordenone, Udine) between January 2008 and July 2010 (Hiwate Project) (Nieuwenhuijsen et al. 2009). Cases were incident histologically confirmed colorectal cancer patients (ICD-10: C18, C19, C20), interviewed as soon as possible after the diagnosis (median of 28 days) through active searches including periodical visits to the hospital departments (i.e. gastroenterology, oncology, general surgery, radiotherapy and pathology). Controls were frequency matched to cases by sex, age (± 5 years), and area of residence, with a case:control ratio of 1. Controls were identified among patients admitted to the same network of hospitals as cases for a wide spectrum of acute, non-neoplastic conditions unrelated to tobacco and alcohol consumption, long-term diet modification and other factors likely related to colorectal cancer. Overall, 52% of controls had acute surgical conditions, 15% orthopedic disorders, 12% dental, ear, nose and throat diseases, and 21% miscellaneous other conditions.

In both countries, study subjects were 20-85 years-old, resided in the hospital catchment area for at least 6 months prior to recruitment, and were able to answer the epidemiological questionnaire. Participant hospitals (17 in Spain, 10 in Italy) were the reference centers for oncologic diseases in each study area. The study protocol was approved by the ethical review board from participating centers and all participants signed an informed consent before recruitment.

Data collection and response rates

Trained interviewers administered questionnaires to collect personal information on socio-demographic factors, lifestyle (smoking, alcohol consumption, physical activity, etc.), anthropometrics (height, weight), residential history, type of water consumed in each residence (municipal, bottled, other), frequency of showering, bathing and hand dishwashing, occupation, medication, medical history and family history of cancer. Amount of water ingested was ascertained as number of glasses per day consumed on average as an adult at home, the workplace and other places, separately for bottled water, municipal water, water from other sources, coffee, tea and herbal drinks. A final section evaluating the quality of the interview was completed by the interviewer. Dietary habits were collected through semi-quantitative food frequency questionnaires that were self-administered in Spain and asked by an interviewer in Italy. Sample size was fixed by the primary scientific objectives of the MCC-Spain Study (Castano-Vinyals et al. 2015). Prior to any analysis we calculated, using the program GRANMO 7.12 (Marrugat et al. 1998), that statistical power was 100% using data from the literature on total THM distribution (Villanueva et al. 2012), assuming a difference of 4 μg/l between cases and controls (based on current preliminary data), and accepting an alpha risk of 0.05 in a two-sided contrast. Response rates were calculated using subjects interviewed in the numerator, and all subjects including refusals in the denominator. Average response rates were 68% in Spain and 95% in Italy among cases, and 53% in Spain and 95% in Italy among controls. In total, 2371 cases (1905 Spain, 466 Italy) and 4159 controls (3590 Spain, 569 Italy) were interviewed.

Trihalomethane levels in the study areas

We used trihalomethanes (THMs) as a surrogate of DBPs. We collected routine THM measurements (chloroform, bromodichloromethane, dibromochloromethane and bromoform)

and historical information on water source (surface/ground proportion) and treatment in the drinking water supplied to the study areas back to 1940 through a structured questionnaire aimed at water utilities, local and health authorities. Routine monitoring data in Spain (2004-2010) were provided by the SINAC (Sistema de Información Nacional en Aguas de Consumo), and by the Regional Environmental Health Agency (Milan) and the Local Health Authority (Pordenone/Udine) in Italy. Availability of THM measurements differed among study areas, with the longest record starting in 1979. A total of 275 water zones in Spain and 370 in Italy were included.

Estimation of long-term trihalomethane levels in drinking water

Annual average levels were calculated for the 4 THMs separately at the water zone level, i.e. the minimum geographic unit with homogeneous water source, treatment and quality (corresponding to municipality in most cases). Cases and controls lived on average in 3 residences during the exposure window, so cases and controls from an area had different and multiple water zones assigned. Years without measurements were assigned the average of all available measurements in the water zone, as long as water source and treatment did not change over the years. Trihalomethane concentrations in surface water are usually higher than in ground sources, and we used surface water percentage as a weight to back extrapolate THM concentrations when water source changed, assuming that concentrations increased proportionally to the percentage of surface water. The same assumptions were used for the 4 THM, and were applied uniformly in all water zones. Hypochlorite was the main disinfectant used in all the study areas. Alternative or complementary treatments identified in the study areas included permanganate, chlorine dioxide and ozonation. Water zones with THM measurements and changes in treatment over the years were used to estimate the change percentage in the 4 THMs separately after introducing such treatments. These percentages

were applied as a weight to back extrapolate concentrations of the 4 THMs separately in areas with changes in these specific treatments for years when measurements were unavailable. Before chlorination started, THM concentrations were assumed to be zero. Year when chlorination started widely varied between study areas, ranging from previous to 1940 (Asturias, Barcelona, Cantabria) to 2005 (a small municipality in Valencia). Following these procedures we estimated annual average THM levels by water zone back to 1940 in areas covering 83% of study person-years, ranging from 67% (León) to 95% (Gipuzkoa).

Individual exposure in the study population

Average THM concentrations in residential tap water (μg/L). Trihalomethane concentrations (the 4 THMs separately) and subjects' personal data were linked by year and water zone of residence to obtain annual concentrations in the residences where subjects lived from age 18 to 2 years before the interview (from now on referred as "lifetime"). Average concentration in all residences with THM estimates was then calculated.

Average THM ingestion (μg/day) was estimated by calculating the average residential THM concentration assuming zero level in residences consuming bottled water (based on Font-Ribera et al. 2010) and 0.3, 0.3, 0.8, and 1.8 μg/l, respectively, for chloroforom, bromodichloromethane, dibromochloromethane, and bromoform in residences consuming private well water. These well water values were the average of 56 observations from water zones with chlorinated ground water in different areas (Gipuzkoa, Barcelona, Valencia and Navarra), as we assumed that private well water was probably chlorinated. Municipal THM concentration was assigned to the years living in residences with municipal water consumption. We averaged the THM concentration calculated following this procedure in residences from age 18 to 2 years before the interview, and multiplied by the amount of water consumed at home (liters/day, assuming 200 ml/glass). We used total water consumed at the

residence (municipal, bottled, private well) because we did not have the specific amount by residence and these habits may have changed over life.

*Showering and bathing THM exposure (μg/l*min/day)* was evaluated as average duration of showers and baths (minutes/week) combined with average THM concentration in residential tap water.

Genotyping

A blood or saliva sample for DNA analysis was collected in all Spanish areas. Response rate among those included in the present analysis was 93%, and DNA was obtained from 3579 blood and 926 saliva samples. Due to budget restrictions, genotyping was conducted in a random sample of subjects (except Murcia area, where genotyping was not conducted), covering 71% of Spanish subjects. Genome-wide genotyping was performed using a customized version of the Infinium Human Exome BeadChip Kit v.1.1 (Illumina). A total of 5363 SNPs of interest for the MCC study were added to the chip, having a total of 248264 markers. The genotype calling was done with the GeneTrain2.0 algorithm (GenomeStudio software) based on CHARGE clusters (Grove et al. 2013). PLINK was used for the genetic data quality control (Purcell et al. 2007) and standard checks were done: sample call rate, sex concordance, heterozigosity, relatedness, population stratification, minimum SNPs call rate of 95% and departure from Hardy-Weinberg equilibrium (HWE) for each SNP tested among Spanish controls. After quality control, the final database included 232396 SNPs with 94659 of them monomorphic and 99411 with a minor allele frequency (MAF) lower than 1%, 8853 with MAF 1–5%, and 29 473 with MAF > 5%. For the present analysis we selected SNPs in *CYP2E1* and *GSTZ1* genes, based on prior work by Cantor et al. (2010). After genotyping quality controls, 22 SNPs in *CYP2E1* and 7 SNPs in *GSTZ1* were available for statistical

analysis (see Table S2 for complete list). Genetic data was available for 981 cases and 2204 controls, which corresponds to a random sample of participants from Spain.

Covariables

Smokers were defined as those smoking at least 100 cigarettes or 360 grams of tobacco in life on a regular basis, i.e. at least a cigarette/day during 6 months or more. Former smokers were defined as those who quit smoking at least one year before the interview. Users of non-steroidal anti inflammatory drugs (NSAIDs) were coded as ever (or never) users if subjects consumed (or not) NSAIDs at least 30 times over life. Physical activity was ascertained through open questions on any type of physical activity practiced in life, the years and the frequency (hours/week), converted to metabolic equivalents (METs) from age 16 (Spain) or 15 (Italy) to 2 years before the interview. Cancer diagnosis (any type) in first degree relatives was asked, and for positive responses the type of cancer was further ascertained. Family history of colorectal cancer was defined by self-reported malignant tumors (polyps excluded). Body mass index was calculated based on the weight one year before the interview and was categorized in 3 groups (<25, 25-29.9, ≥ 30 kg/cm^2). A total of 156 food items were ascertained through a food frequency questionnaire, assessing usual dietary intake the year before the interview. Frequencies in servings/day of red meat, processed meat, dairy products, fruits and vegetables were converted to grams/day based on food composition tables.

Statistical analysis

Only subjects with average THM concentrations in the residential tap water, average THM ingestion and showering/bathing THM estimates for at least 70% of the years between age 18 to 2 years before the interview (89% of interviewed subjects) were considered in the

respective models, and subjects with unreliable interviews (N=16) were excluded. This led to a total of 5765 subjects for the statistical analyses (2047 cases, 3718 controls), of which 5291 (1837 cases, 3454 controls) were analyzed for average THM concentrations in residential tap water and 5731 (2047 cases, 3684 controls) for ingested THMs. The higher numbers for the ingestion variable are explained by those whose residential THM concentration cannot be estimated and consumed bottled or private well water. We used unconditional logistic regression to estimate odds ratio (OR) and 95% confidence intervals (CI) for associations between colorectal cancer and THM exposures adjusted for age (continuous), sex, area (as a factor in the model), education, smoking, use of non-steroidal anti inflammatory drugs, leisure physical activity, and family history of colorectal cancer (first degree relatives), categorized as shown in Table 1. Missing values in categorical variables were coded as a separate category. There were no missing values for the matching variables (age, sex, area). Categories of body mass index, and mg/day (continuous) of red meat, processed meat, dairy products, fruits and vegetable consumption were explored but did not meet our criterion for confounding (<10% change in the risk estimates) and were omitted to keep the most parsimonious models. Given different genotoxicity (Landi et al. 1999) and collinearity among THMs, analyses were conducted separately for chloroform and brominated THMs (bromodichloromethane, dibromochloromethane and bromoform), and mutually adjusted ORs for both exposures were not estimated. Exposure categories were created according to quartiles of THM exposure overall, for both cases and controls. Linear p-trends were calculated using likelihood-ratio test comparing the model with and without the exposure variable with quartiles coded numerically (0, 1, 2, 3). Generalized additive models (GAM) were used to evaluate the exposure-response relationships on continuous variables, using a smoothed spline with 3 degrees of freedom using *gam* function[a] with Stata Statistical software, release 12. Departure from linearity was assessed by testing the difference in

normalized deviance between the GAM and a model with a linear term for the exposure, based on a chi-squared approximation. Other exposure variables examined included the type of water consumed at the longest residence, and showering/bathing frequency combined with THM concentrations (min/day*μg/L). The main analyses were stratified by sex given the higher incidence among men, expected differences in established risk factors and for comparability with previous studies. We explored potential differences by cancer site (colon, rectum) in stratified analyses for comparability with previous studies. P-value threshold to define statistical significance was set at <0.05. Interactions between lifetime average THM concentrations at the residential tap (dichotomized at percentile 75) and genotypes were examined to assess whether odds ratios within genotype categories by using the dominant model (heterozygous and homozygous variant versus homozygous wild type). We used the likelihood-ratio test to evaluate the joint effect of the SNP and the interaction term. Linkage disequilibrium was evaluated and the number of effective tests with a r2 cut-off set at 0.8 was 15. Bonferroni corrections for number of effective tests were applied for genotype analyses.

RESULTS

A total of 5765 subjects were included in the present analysis, 1647 cases and 3215 controls from Spain, and 400 cases and 503 controls from Italy (Table 1). The area contributing with the largest population was Barcelona (28% of all subjects) followed by Madrid (15%). The proportion of men was higher among cases (64%) than in controls (52%). Cancer location was colon among 1410 (69% cases), rectum among 607 (30%) and unspecified or missing among 30 (1%). Proportion of smokers, BMI above 25 Kg/m^2, and family history of colorectal cancer was higher among controls while proportion of NSAID users was higher among controls and physical activity was similar among cases and controls (Table 1). Water

type consumed in the longest residence, residential history and THM exposure is described in Table 1.

Concentration and composition of trihalomethanes differed between study areas (Figure 1). Median concentrations overall were 29.5, 17.4, and 9.0 μg/l, respectively for residential total THMs, chloroform and brominated THMs. Area median concentration of total THMs ranged from 1 μg/L in Udine/Pordone, Italy, to 86 μg/L in Barcelona. Brominated THMs were highest in Murcia and Barcelona, with a median of 65 and 64 μg/L, respectively and chloroform was highest in Madrid (median 28 μg/L). Differences of ingested THMs among areas was less pronounced than concentrations, since the areas with higher concentrations usually have higher proportion of bottled water consumption. The Spearman correlation coefficient between total THMs vs. chloroform, bromodichloromethane, dibromochloromethane and bromoform, was, respectively, 0.68, 0.87, 0.77 and 0.64. Between total brominated THMs and bromodichloromethane, dibromochloromethane and bromoform, it was, respectively, 0.96, 0.98, 0.90. These correlations varied among study areas. Total THM-chloroform correlation was above 0.84 in all areas except in Barcelona (-0.32). The distribution density of average lifetime THM concentrations in the residential tap water by case-control status and area is shown in Figure S1.

Lifetime average total THM concentrations were not positively associated with colorectal cancer among men or women while a negative association was observed for some exposure categories at the low exposure range (Table 2). Chloroform concentrations were negatively associated with colorectal cancer, with strongest associations for the highest versus lowest quartile both among men and women. Brominated THM concentrations were positively associated with colorectal cancer among men in the highest versus lowest exposure category

(OR=1.43, 0.83-2.46) and negatively associated in the third versus lowest quartile (OR=0.57, 0.36-0.90) and among women (Table 2). Generalized additive models confirmed these patterns, revealing non-linear exposure-response relationships (Figure 2). However, the curves have a low precision at the high end of the exposure ranges and estimates are based on small numbers. Exposure-response curves for the 4 THMs, men and women combined, are showed in the Figure S2. Approximately linear negative associations are observed for chloroform and positive associations for bromoform, while for bromodichloromethane and dibromochloromethane associations are non-linear. Associations by area showed heterogeneity, with Barcelona showing similar results to the overall estimates (Figure S3 and Figure S4). Conversely, models with all areas except Barcelona show attenuated dose-response relationships (Figure S3 and Figure S4), showing that Barcelona, with ~26% of all controls and ~31% of all cases and observations in highest quartiles of brominated THMs, drives the overall results.

Interactions between exposure to total and brominated THMs and 22 selected polymorphisms were evaluated. Nominal statistically significant interactions were observed between four SNPs in the *CYP2E1* gene (rs2070675, rs915907, rs8192775, rs743535) in relation to colorectal cancer and exposure to brominated THMs at concentrations above 40 micrograms/L (Table 3), however, none of them was statistically significant according to the Bonferroni corrected p-value (0.03). Due to changes in the reference category and the sample with genetic data, the OR for the highest quartile versus quartiles 1-3 combined are ~2–4 for brominated and total THMs (Table 3). Results for all SNPs analyzed in relation to different exposure cut-offs for total, chlorinated and brominated THMs are shown in Supplementary Table, S2).

The use of bottled water in the longest residence (33 years long on average) versus municipal water was positively associated with colorectal cancer, OR=1.19 (1.04-1.37) among men and women combined, OR=1.17 (0.98-1.39) among men, and OR=1.20 (0.96-1.50) among women. Ingested chloroform level was negatively associated with colorectal cancer among men and women (Table 4). Ingested brominated THMs led negative associations among women, and men in quartiles 2 and 3 vs. 1, while a positive association was observed among men in quartile 4 vs. 1 (Table 4). Generalized additive models (GAM) confirmed these patterns (See Figure S5), although the curves at the high end of the exposure range have a low precision and estimates are based on small numbers.

When shower-bath duration was combined with THM concentrations, positive associations were observed for the highest exposure to brominated THMs among men and null to protective associations for chloroform (Table 5 and Figure S6).

By cancer site, associations for colon and rectal cancer evaluated separately showed similar patterns particularly among men, while slight differences appeared among women (see Table S3). However, a majority of cases (69%) are colon cancers, and numbers of cases of rectal cancer are especially small among women, thus leading to imprecise estimates.

DISCUSSION

We estimated the long-term exposure to four trihalomethanes in drinking water in a large multicentric case-control study of colorectal cancer, including areas with contrasting THM concentrations and evaluating different routes of exposure. In accord with some experimental evidence, chloroform concentrations were inversely associated with colorectal cancer in men and women. A positive association with colorectal cancer was only identified in the highest

exposure category of brominated THM concentrations among men. Similar associations were found for colon and rectal cancer analyzed separately.

Evidence of DBP carcinogenicity in animals differs by DBP chemical, animal species, organ and administration route. Increased incidence of preneoplastic lesions were found in the colon of rats exposed to chloroform, bromodichloromethane, dibromochloromethane, bromoform, and MX, individually and in mixtures (McDorman et al. 2003), but has not been found in mice (DeAngelo et al. 2002). Bromodichloromethane administered by gavage was the trihalomethane causing the widest spectrum of neoplasms in rodents, including the large intestine (Dunnick et al. 1987). However, another study found resistance of the intestinal tract to the effects of MX (3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone) on preneoplastic or neoplastic development, despite the elevated MX genotoxicity observed in the gastrointestinal (GI) tract (Steffensen et al. 1999). Specific evidence on gastrointestinal carcinogenicity has shown that chloroform inhibits chemically induced tumors (Daniel et al. 1989) and nuclear anomalies (Daniel et al. 1991) in rodents exposed to GI carcinogens. More recently, chloroform was identified as the active component of an herbal drug used to treat cancer (Zhang et al. 2014; Yu et al. 2007), and chloroform fraction of a traditional drug used in Asia has shown antitumor activity (Habib et al. 2011). Although mechanisms are not known, a possible explanation would involve destroying selectively the initiated cells (Reddy et al. 1992) through apoptosis and proliferation suppression (Zhang et al. 2014). Finally, the association with chloroform should be cautiously interpreted and remains to be confirmed.

Approximately 11% of the study population had exposures above the maximum contaminant levels for total THMs in Spain (100 μg/l), with the maximum concentration observed at 174 μg/l, most of them clustered in the area of Barcelona (97%) followed by Murcia (3%). In Italy, all study subjects were below the regulatory limits (30 μg/l). Specific THMs are not

regulated in Italy or Spain (BOE 2003; GURI 2001). The highest exposure categories defined in previous studies were ≥75 μg/l (King et al. 2000), ≥46.4 μg/l (Hildesheim et al. 1998) and >40 μg/l (Young et al. 1987), included in the range of our study. The case-control study by Bove, Jr. et al. (2007), including only men, shows a median chloroform concentration (17,6 μg/l) similar to our median concentration (17 μg/l). Exposure periods are comparable with previous studies, that covered either lifetime (Hildesheim et al. 1998; Young et al. 1987) or 40 years before the interview (King et al. 2000). However, the present study evaluates populations with higher concentrations of brominated THMs compared to previous case-control or cohort studies evaluating colorectal cancer. Only one case-control study evaluated associations for brominated THMs (Bove, Jr. et al. 2007), showing positive associations of rectal cancer. Our results are not consistent with the cohort study conducted by Doyle et al. (1997). The predominant THM was chloroform (geometric mean 46.1 μg/l in surface waters, 82% from total THM) and associations were estimated for this substance. They reported a positive association for colon cancer (RR 1.68, 1.11-2.53), and a null association for rectal cancer (RR 1.07, 0.60-1.93) for the highest exposure category (14-287 μg/l). To our knowledge, we present the first epidemiological study reporting a negative association with chloroform. However, King et al. (2000) reported negative associations for some categories of total THM exposure among women, without a clear dose-response. Our findings showing different associations for specific THMs lead us to speculate that the use of total THMs could at least partly explain the heterogeneous results in previous studies, by disregarding different DBP compositions. Finally, the gut microbiota may play a role in the colorectal carcinogenesis, as chlorinated water has been shown to alter the enteric environment in mice (Sasada et al. 2015). This has received recently attention and evidence is limited.

While negative associations with chloroform are found both for men and women, a positive association with brominated and total THMs is found among men at the high end of the

exposure range, where there are few observations and precision is low. Similarly, previous studies have found inconsistent differences in colorectal cancer associated with THM exposure between men and women (King et al. 2000). Although associations were minimally modified by introducing covariables in the models (data not shown), residual confounding cannot be ruled out.

Exposure measurement error is a concern in cancer studies, given the long exposure windows and limited historical THM measurements. In this study we applied different strategies to minimize measurement error in the exposure. Subjects included in the models had exposure estimates covering a major portion of the exposure window (at least 70%). Since quality of the interview is related to measurement error (Villanueva et al. 2009), we only analyzed subjects with reliable interviews. Finally, inability to account for THM exposure outside the home may have introduced error in the ingestion THM estimates, although most of total water was consumed at home (74%).

We cannot rule out uncontrolled confounding by other water contaminants. However, long-term exposure assessment to nitrate has been conducted following comparable methods to trihalomethanes (Espejo-Herrera et al. 2016). Lifetime average nitrate concentrations in the residence ranged approximately from 3 to 20 mg/l, and Pearson correlation coefficient between long-term nitrate and THMs concentrations was 0.03 (range 0.10 to -0.76) for total THMs, -0.41 (range -0.11 to -0.56) for chloroform and 0.15 (-0.05 to -0.72) for brominated THMs overall (minimum-maximum per area). In order to explore potential confounding by nitrate, we adjusted our main model additionally for nitrate. The point estimates varied minimally (data not shown), ruling out a major confounding effect of nitrate.

Selection bias might be a concern, particularly in Spain where response rates were lower compared to Italy. The high response rate in Italy reflects a traditionally high compliance of

subjects approached for interview in the study hospital-based case-control network. In addition, controls are population-based in Spain while hospital-based in Italy, explaining lower response rates among controls in Spain. However, we assume that probability of participation is independent from the exposure, and we don't expect an impact on the results due to response rates. Study subjects in Spain are part of a larger study where a common pool of controls is shared for different cancer sites (colorectal, breast, prostate, stomach, lymphocytic chronic leukemia), leading a larger number of controls than cases and slightly different age and sex distribution.

Metabolism of DBPs is mediated by enzymes from the glutathionine S-transferase (GST) and cytochrome P450 (CYP) families (DeMarini et al. 1997; Pegram et al. 1997; Raucy et al. 1993). We were able to examine interactions between THM exposure and polymorphisms in two of the three genes that modified associations between THMs and bladder cancer in a previous study (Cantor et al. 2010). We found an association with polymorphisms in the *CYP2E1* that were not significant after correction for multiple comparisons. We did not find indications that polymorphisms in *GSTZ* modify the effects of the exposure to brominated THMs. Even though we evaluated gene–environment interactions in a large study, the analysis was limited by dichotomization of exposure and we still have limited power to detect modest interactions after controlling for multiple comparisons.

CONCLUSIONS

Associations with colorectal cancer differed for estimated exposures to chloroform versus brominated THMs in residential drinking water in our case-control study. Findings suggest a protective effect of chloroform while a positive association with brominated THMs is observed among men at the extreme tail of the exposure distribution where results may be driven by small number of observations. Overall, these results require confirmation.

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Table 1. Characteristics of the study population, 2047 cases of colorectal cancer and 3718 controls.

Variable		Cases	Controls
Age (years), mean (SD)		67 (10)	64 (11)
		n (%)	*n (%)*
Sex			
	Men	1317 (64.3)	1943 (52.3)
	Women	730 (35.7)	1775 (47.7)
Area			
	Asturias (Spain)	70 (3.4)	211 (5.7)
	Barcelona (Spain)	635 (31.0)	957 (25.7)
	Cantabria (Spain)	125 (6.1)	282 (7.6)
	Guipuzcoa (Spain)	113 (5.5)	329 (8.9)
	León (Spain)	294 (14.4)	360 (9.7)
	Madrid (Spain)	204 (10.0)	670 (18.0)
	Murcia (Spain)	28 (1.4)	39 (1.1)
	Navarra (Spain)	102 (5.0)	234 (6.3)
	Valencia (Spain)	76 (3.7)	133 (3.6)
	Milano (Italy)	215 (10.5)	301 (8.1)
	Pordenone/Udine (Italy)	185 (9.0)	202 (5.4)
Education			
	< Primary school	519 (25.4)	592 (15.9)
	Primary school	766 (37.5)	1234 (33.2)
	Secondary school	566 (27.7)	1182 (31.8)
	University	190 (9.3)	707 (19.0)
	Missing	6	3
Smoking			
	Never	841 (41.4)	1629 (43.9)
	Former	821 (40.4)	1290 (34.8)
	Current	371 (18.2)	789 (21.3)
	Missing	14	10
Non steroidal anti inflammatory drugs consumption[a]			
	No	1382 (69.7)	2237 (62.3)
	Yes	600 (30.3)	1352 (37.7)
	Missing	65	129
Body mass index (kg/cm^2)[b]			
	<25	676 (33.0)	1391 (37.8)
	25-29.9	909 (44.4)	1555 (42.3)
	≥30	461 (22.5)	734 (20.0)
	Missing	1	38
Leisure physical activity (adult life average)[c]			
	0 METs	627 (30.6)	1011 (27.4)
	0-8 (8.5) METs	698 (34.1)	1324 (35.9)
	8-16 (34.4) METs	294 (14.4)	582 (15.8)
	>16 (34.4) METs	428 (20.9)	767 (20.8)

Missing	0	34
Family history colorectal cancer (first degree)		
No	1648 (85.4)	3246 (92.0)
Yes	281 (15.6)	284 (8.0)
Missing	118	188
Drinking water type at the longest residence		
Tap (municipal)	1185 (58%)	2474 (67%)
Bottled	655 (32%)	1014 (27%)
Private well	198 (10%)	227 (6%)
Missing	9	3
	Mean (SD)	Mean (SD)
Number of residences	3.2 (1.5)	3.3 (1.6)
Years at the longest residence	35.6 (12.0)	31.8 (12.1)
Average THM concentrations in residential tap water (μg/L)[b]	44.8 (43.9)	40.3 (34.2)
Average THM ingestion from residential tap water (μg/day)	28.1 (48.8)	25.9 (36.4)
Average showering/bathing THM concentrations (min/day*μg/L)[c]	284.7 (406.4)	266.6 (311.5)

The table excludes subjects with inadequate THM exposure data and 16 observations with low quality interviews.
[a] Ever users consumed non steroidal anti inflammatory drugs at least 30 times in life.
[b] Body mass index based on the weight one year before the interview.
[c] METs, metabolic equivalents. Categories were country-specific and cut offs for Italy are in parenthesis.
[b] Number of observations: 1977 cases, 3679 controls
[c] Number of observations: 1834 cases, 3480 controls

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Table 2. Association between average trihalomethane (THM) concentrations in the residential tap water and colorectal cancer. Odds ratio (OR) and 95% confidence intervals (CI) among 1837 cases and 3454 controls.

Exposure	cases	cont.	OR[a] (95% CI)	cases	cont.	OR[a] (95% CI)	cases	cont.	OR[a] (95%CI)
			All (N=5291)			Men (N=2977)			Women (N=2312)[b]
Total THMs (μg/l)									
<16.2	591	741	1	393	447	1	196	294	1
16.2-29.5	378	954	0.57 (0.45, 0.72)	241	407	0.56 (0.41, 0.77)	137	547	0.62 (0.41, 0.93)
29.5-62	323	1007	0.44 (0.33, 0.58)	193	530	0.38 (0.27, 0.55)	130	477	0.60 (0.38, 0.93)
>62	545	752	0.92 (0.66, 1.28)	349	417	1.04 (0.68, 1.60)	196	335	0.68 (0.39, 1.20)
p-trend[c]			*0.187*			*0.741*			*0.165*
Chloroform (μg/l)									
<6	589	744	1	387	446	1	200	298	1
6-17.4	435	895	0.69 (0.55, 0.87)	268	404	0.69 (0.51, 0.93)	167	491	0.71 (0.49, 1.03)
17.4-23.4	515	798	0.68 (0.53, 0.87)	335	384	0.73 (0.53, 1.01)	180	414	0.65 (0.43, 0.97)
>23.4	298	1017	0.31 (0.24, 0.41)	186	567	0.26 (0.18, 0.37)	112	450	0.46 (0.29, 0.72)
p-trend[c]			*<0.001*			*<0.001*			*0.001*
Brominated THMs (μg/l)									
<3.7	541	792	1	348	483	1	191	309	1
3.7-9.0	387	943	0.77 (0.60, 0.98)	238	419	0.73 (0.53, 1.01)	149	524	0.78 (0.52, 1.17)
9.0-41.8	368	963	0.55 (0.38, 0.79)	241	479	0.57 (0.36, 0.90)	127	484	0.49 (0.26, 0.91)
>41.8	541	756	1.00 (0.65, 1.53)	349	420	1.43 (0.83, 2.46)	192	336	0.44 (0.21, 0.92)
p-trend[c]			*0.556*			*0.036*			*0.039*

[a]Adjusted for (sex), age, area, education, non steroidal anti inflammatory drugs consumption, smoking, physical activity and family history of colorectal cancer. The analysis excludes subjects with poor or questionable interview and with known THM concentrations for less than 70% of the exposure window (from age 18 to 2 years before the interview).

[b] Two women cases are lost from the sex-stratified model because one category in education does not have observations among controls and is dropped from the model.

[c] Linear trend p-value, derived from a likelihood ratio test comparing a model with the categorical nitrate variable as an ordinal variable (0, 1, 2), with a model that excluded the variable.

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Table 3. Interaction between polymorphisms in *CYP2E1* and trihalomethanes (THMs) exposure, dichotomized at the percentile 75. Odds ratio (OR) and 95% confidence intervals (CI) among 981 cases and 2204 controls.

Gene, SNP [a]	Genotype	cases	controls	Total THMs (> vs. ≤ 60 μg/l)		Brominated THMs (> vs. ≤ 40 μg/l)		Chloroform (> vs. ≤ 20 μg/l)	
				OR (95%CI)[b]	Nominal *p* value[c]	OR (95%CI)[b]	Nominal *p* value[c]	OR (95%CI)[b]	Nominal *p* value[c]
rs2070675	CC	682	1496	3.5 (2.4,5.1)	0.029	4.1 (2.7,6.0)	0.009	0.4 (0.3,0.6)	0.646
	CT/TT	298	707	2.3 (1.3,4.0)		2.1 (1.2,3.7)		0.6 (0.4,0.9)	
rs915907	CC	710	1566	3.3 (2.2,4.7)	0.031	3.9 (2.7,5.8)	0.009	0.4 (0.3,0.5)	0.392
	AC/AA	252	618	2.6 (1.4,5.0)		2.3 (1.2,4.3)		0.8 (0.5,1.2)	
rs8192775	GG	850	1884	3.1 (2.2,4.4)	0.103	3.7 (2.6,5.3)	0.021	0.5 (0.4,0.6)	0.383
	AG/AA	131	320	2.7 (1.1,6.5)		1.7 (0.8,3.9)		0.5 (0.2,1.0)	
rs743535	GG	838	1823	3.0 (2.1,4.3)	0.056	3.7 (2.6,5.3)	0.019	0.5 (0.3,0.6)	0.066
	AG/AA	142	381	3.4 (1.5,7.8)		2.0 (0.9,4.3)		0.5 (0.3,0.9)	

SNP, Single nucleotide polymorphism.

[a] Results for all SNPs are reported in table S2.

[b] Adjusted for (sex), age, geographical area and education.

[c] Likelihood ratio test (LRT) for the joint effect of the SNP and interaction term. The critical *p* value with Bonferroni corrections is $p<0.003$.

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Table 4. Association between average trihalomethanes (THMs) ingestion in the residences and colorectal cancer. Odds ratio (OR) and 95% confidence intervals (CI) among 2047 cases and 3684 controls.

Exposure	cases	cont.	OR[a] (95% CI)	cases	cont.	OR[a] (95% CI)	cases	cont.	OR[a] (95%CI)
			All (N=5731)			Men (N=3239)			Women (N=2490)[b]
Total THMs (μg/day)									
<1.3	601	836	1	390	491	1	209	345	1
1.3-12.2	555	882	0.98 (0.83, 1.17)	372	448	0.99 (0.79, 1.25)	183	434	1.04 (0.78, 1.39)
12.2-32.3	395	1039	0.74 (0.61, 0.91)	245	474	0.74 (0.58, 0.96)	150	565	0.83 (0.60, 1.14)
>32.3	496	927	0.83 (0.68, 1.00)	310	509	0.82 (0.64, 1.05)	186	418	0.86 (0.63, 1.17)
p-trend[c]			*0.008*			*0.034*			*0.176*
Chloroform (μg/day)									
<0.2	621	816	1	403	471	1	216	345	1
0.2-5.3	537	897	0.87 (0.74, 1.03)	356	489	0.86 (0.70, 1.05)	181	408	0.98 (0.75, 1.29)
5.3-16.6	477	955	0.82 (0.69, 0.98)	310	435	0.89 (0.71, 1.12)	167	520	0.78 (0.58, 1.04)
>16.6	412	1016	0.67 (0.56, 0.82)	248	527	0.63 (0.49, 0.81)	164	489	0.74 (0.54, 1.01)
p-trend[c]			*<0.001*			*0.001*			*0.031*
Brominated THMs (μg/day)									
<0.7	590	847	1	384	496	1	204	351	1
0.7-3.7	521	918	1.04 (0.86, 1.26)	332	467	0.99 (0.78, 1.26)	189	451	1.26 (0.91, 1.74)
3.7-11.9	412	1024	0.79 (0.65, 0.96)	262	460	0.76 (0.59, 0.98)	150	564	0.85 (0.61, 1.19)
>11.9	524	895	0.83 (0.68, 1.02)	339	499	0.88 (0.68, 1.14)	185	396	0.83 (0.60, 1.15)
p-trend[c]			*0.007*			*0.098*			*0.059*

[a]Adjusted for (sex), age, area, education, smoking, physical activity, non-steroidal anti inflammatory drug consumption, family history of cancer. Models exclude subjects with poor or questionable questionnaires and with THM ingestion estimated for less than 70% of the exposure window (from age 18 to 2 years before the interview).

[b] Two women cases are lost from the sex-stratified model because one category in education does not have observations among controls and is dropped from the model. [c] Linear trend p-value, derived from a likelihood ratio test comparing a model with the categorical nitrate variable as an ordinal variable (0, 1, 2), with a model that excluded the variable.

Table 5. Association between colorectal cancer and shower-bath THM levels. Odds ratios (OR) and 95% confidence intervals (CI) among 1702 cases and 3269 controls.

Exposure	cases	cont.	OR[a] (95% CI)	cases	cont.	OR[a] (95% CI)	cases	cont.	OR[a] (95%CI)
			All (N=4971)			Men (N=2793)			Women (N=2176)[b]
Total THMs (μg/l*min/day)									
<60.7	550	701	1	363	444	1	185	257	1
60.7-158.7	382	867	0.72 (0.58,0.89)	244	420	0.76 (0.58,0.99)	138	447	0.67 (0.47,0.97)
158.7-336.7	329	920	0.60 (0.48,0.76)	203	416	0.68 (0.50,0.92)	126	504	0.54 (0.37,0.80)
>336.7	441	781	0.81 (0.63,1.06)	279	424	0.92 (0.65,1.29)	162	357	0.71 (0.46,1.09)
p-trend[c]			*0.121*			*0.426*			*0.141*
Chloroform (μg/l*min/day)									
<26.1	550	701	1	361	435	1	188	266	1
26.7-72.5	444	804	0.85 (0.70,1.03)	284	405	0.90 (0.70,1.16)	159	399	0.82 (0.59,1.13)
72.5-143.5	373	861	0.65 (0.53,0.81)	237	445	0.68 (0.51,0.89)	136	416	0.68 (0.48,0.97)
>143.5	335	903	0.66 (0.52,0.83)	207	419	0.73 (0.54,0.99)	128	484	0.60 (0.41,0.88)
p-trend[c]			*<0.001*			*0.016*			*0.007*
Brominated THMs (μg/l*min/day)									
<16.5	539	712	1	345	455	1	192	257	1
16.5-51.0	339	911	0.66 (0.53,0.83)	217	431	0.76 (0.57,1.01)	122	480	0.52 (0.36,0.74)
51.0-178.0	345	904	0.61 (0.47,0.80)	220	401	0.78 (0.56,1.09)	125	503	0.43 (0.28,0.65)
>178	479	742	0.90 (0.65,1.25)	307	417	1.25 (0.83,1.89)	172	325	0.55 (0.32,0.95)
p-trend[c]			*0.267*			*0.627*			*0.010*

[a]Adjusted for (sex), age, geographical area, education, non steroidal anti inflammatory drugs consumption, smoking, physical activity and family history of colorectal cancer. The analysis excludes subjects with poor or questionable interview and with known THM levels for less than 70% of the exposure window (from age 18 to 2 years before the interview).

[b] Two women cases are lost from the sex-stratified model because one category in education does not have observations among controls and is dropped from the model.

[c] Linear trend p-value, derived from a likelihood ratio test comparing a model with the categorical nitrate variable as an ordinal variable (0, 1, 2), with a model that excluded the variable.

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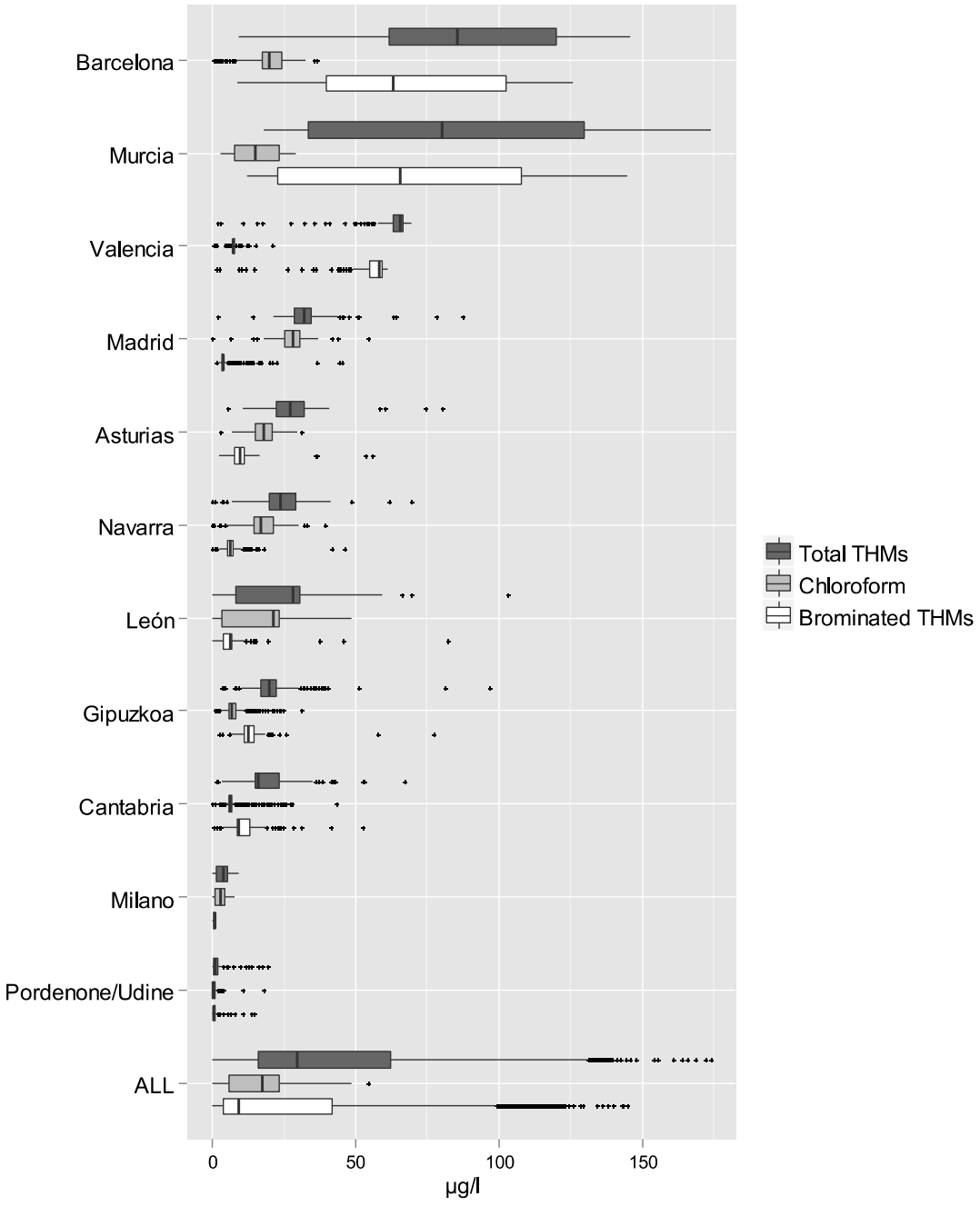
Figure 1. Distribution of average residential trihalomethane (THM) exposure among subjects with THM estimates at least 70% of the exposure window (age 18 to 2 years before the interview) from reliable interviews. Panel A) Average concentrations at the tap (μg/l), N=5291. Panel B) Average ingested levels (μg/day), N=5731. All areas correspond to Spain except Milano and Pordenone/Udine (Italy). Boxes are delimited by the 25th (left hinge) and 75th (right hinge) percentiles, the central vertical line represents the median value, the whiskers represent ± 1.5 times the interquartile range, and the points outside the whiskers represent outliers.

Figure 2. Exposure-response relationship between residential trihalomethane (THM) concentrations (X axis, in μg/l) and colorectal cancer (Y axis, expressed in odds ratios, OR) among 1837 cases and 3454 controls. Panel A) Men; Panel B) Women.

Foot note: Smoothed spline with 3 degrees of freedom from general additive models adjusted for sex, age, area, education, smoking, physical activity, non-steroidal anti inflammatory drugs, family history of cancer. Subjects with unsatisfactory questionnaires and subjects with THM estimated less than 70% from the exposure window are excluded. P-value gain compared to linearity is <0.001 for all models, except for chloroform in women (p-value=0.38). Tick marks above the x-axes represent observations, and the dashed lines represent the 95% confidence intervals.

Figure 1.

A)


Barcelona
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Madrid
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Navarra
León
Gipuzkoa
Cantabria
Milano
Pordenone/Udine
ALL
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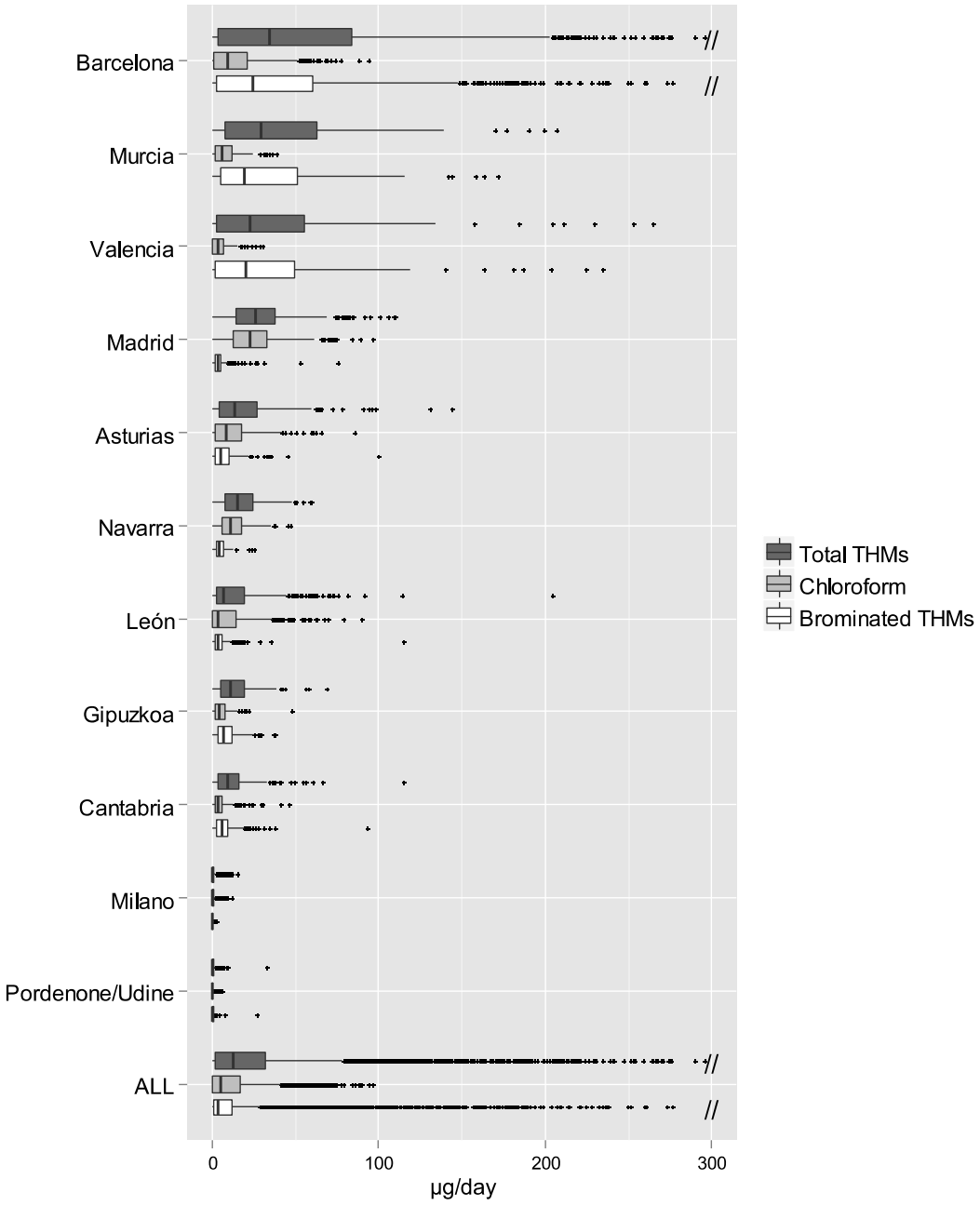
50
100
150
µg/l
Total THMs
Chloroform
Brominated THMs

B)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Milano
Pordenone/Udine
ALL
0
100
200
300
µg/day
Total THMs
Chloroform
Brominated THMs

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Figure 2.