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# Exposure to widespread drinking water chemicals, blood inflammation markers, and colorectal cancer

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## ABSTRACT

*Background:* Trihalomethanes (THMs) and nitrate are widespread chemicals in drinking water associated with colorectal cancer risk but mechanisms are not well understood.

*Objectives:* We explored the association between exposure to THMs and nitrate in drinking water and inflammation markers, and the link with colorectal cancer risk.

*Methods:* A subset of 198 colorectal cancer cases and 205 controls from the multicase-control study MCC-Spain were included. Average concentration of THMs (chloroform, bromodichloromethane, dibromochloromethane, bromoform) and nitrate in tap water at the residence was estimated from age 18 until 2 years before the interview ("long term") and for a recent period (3 years before diagnosis). Serum levels of EGF, eotaxin, G-CSF, IL-17E, IL-1rA, IL-8, IP-10, MDC, MPO, periostin, VEGF, and C-reactive protein (CRP) were measured. We estimated the linear association between inflammation markers and exposure among controls, and the odds ratio of colorectal cancer associated with THM and nitrate exposure, and inflammation markers. A mediation analysis was conducted to identify inflammation markers in the pathway between THM/nitrate exposure and colorectal cancer.

*Results:* Serum concentrations of EGF, IL-8, IL-17E and eotaxin increased with recent residential levels of brominated THMs, chloroform and/or total THM. No associations were observed for nitrate and for long-term residential THM levels. All residential exposures except chloroform were positively associated with colorectal cancer. Serum concentrations of VEGF and periostin were positively associated with colorectal cancer, while EGF was inversely associated. One protein-exposure combination (periostin-recent ingested brominated THMs) slightly mediated the association with colorectal cancer risk.

*Discussion:* Results suggest that estimated THM exposure is involved in inflammation processes. However, the study design was limited to stablish etiologically relevant associations between the protein levels and colorectal cancer risk. The lack of association between nitrate exposure and inflammation markers suggests other biological mechanisms are involved in the link with colorectal cancer.

# 1. Introduction

Colorectal cancer is one of the most common cancers in men and women, representing approximately 10% of the global cancer incidence, with increasing trends in most countries (Stewart and Wild 2014). In 2020, colorectal cancer ranked the third most common cancer with 1.2 million new cases, accounting for 10% of all cancers (Sung et al., 2021). More than 65% of new cases occur in countries with high or very high levels of human development, and almost half of new cases occur in Europe and the Americas (Bosman et al., 2014). Diet is the main etiological factor, with intake of total energy, red and processed meat, alcohol, in addition to physical inactivity, body and abdominal fatness and adult height being established risk factors (Bosman et al., 2014; WCRF and AICR 2011). Protective factors include dietary fiber and high fruit and vegetable consumption (Bradbury et al., 2014; WCRF and AICR 2011). Environmental exposures including disinfection by-products (DBPs) and nitrate in drinking water have been suggested as risk factors for colorectal cancer.

Disinfection by-products (DBPs) are generated during treatment of drinking water, through reaction of disinfectants such as chlorine with organic matter naturally present in raw water (Richardson et al., 2007). DBPs form a complex mixture of >700 chemicals, and trihalomethanes (THMs) are usually the most prevalent and have been used as markers of DBP exposure in epidemiological studies. THMs can be incorporated through ingestion, inhalation and dermal contact, and long-term exposure has been linked to colorectal cancer (Jones et al., 2019; Rahman et al., 2010; Villanueva et al., 2017). The combined relative risk (RR) estimated in a meta-analysis of 13 studies was 1.27 (95% CI 1.08–1.50) for colon cancer and 1.30 (95% CI 1.06–1.59) for rectal cancer, for the highest vs. lowest DBP exposure categories, with slightly higher RR for case-control vs. cohort studies (Rahman et al., 2010). A multicase-control study conducted in Spain in 2008–2013 including 2,047 colorectal cancer cases and 3,718 controls, found no clear evidence of an association between lifetime total THM exposure and CRC (Villanueva et al., 2017). However, a negative association was found with chloroform and a positive association with brominated THMs, suggesting differences among specific THMs (Villanueva et al., 2017). Jones et al. published in 2019 a positive association between rectal cancer risk and exposure estimates of ingested total THM (hazard ratio$_{Q5vsQ1}$ = 1.71, 95% CI 1.00–2.92), bromodichloromethane (HR$_{Q4vsQ1}$ = 1.89, 95% CI 1.17–3.00), and trichloroacetic acid (HR$_{Q4vsQ1}$ = 1.92, 95% CI 1.20–3.09), but not for colon cancer (Jones et al., 2019). In summary, there is extensive suggestive evidence of an increased risk of colorectal cancer and DBP exposure, but heterogeneous associations for specific exposures, populations, cancer sites and/or effect modifiers (Benmarhnia et al., 2018), as well as the lack of understanding of the biological mechanisms prevent a causal inference.

Nitrate is a widespread chemical in drinking water coming from fertilizers, intensive farming and sewage (Wakida and Lerner 2005; Ward et al., 2005). Ingested nitrate is a probable human carcinogen in conditions of endogenous nitrosation (IARC 2010). Long-term exposure to nitrate in drinking water has been consistently linked to colorectal cancer (Ward et al., 2018), and many studies reported increased risks at exposure levels below the regulatory limits (Ward et al., 2018). A multicase-control study conducted in Spain in 2008–2013 including 1,869 cases and 3,530 controls identified and odds ratio (OR) of colorectal cancer of 1.49 (95% CI 1.24–1.78) for long-term exposure to >10 vs. ≤5 mg/day of nitrate (Espejo-Herrera et al., 2016). However, Jones et al. (2019) did not find an association between ingested nitrate in drinking water and colon or rectal cancer risk in the Iowa Women's Health Study cohort (Jones et al., 2019). In addition, Jones et al. (2019) have been the first to investigate simultaneously the exposure to DBPs and nitrate in drinking water associated with risk of colon and rectal cancer, finding no evidence of interaction between total THMs and nitrate on the risk of either cancer site (Jones et al., 2019).

Inflammation is a critical component of carcinogenesis (Hanahan and Weinberg 2011), and colorectal cancer is an heterogeneous disease that develops through a multiple sequence of events, including chronic inflammation (Grizzi et al., 2018). Inflammation is the response of the immune system to infection, injury, and harmful exposures. Among other events, the immune cells produce cytokines, which are a broad group of small proteins involved in cell signaling (Opal and DePalo 2000; Steinke and Borish 2006). THMs and specifically chloroform, bromodichloromethane, dibromochloromethane and bromoform have shown to induce a dose-dependent decreased cell-mediated immunity in rodents sub-chronically exposed (Munson et al., 1982). A recent global transcriptional analysis in human intestinal epithelial cells showed notable effects on transcription genes related to immunity and inflammation after exposure to a range of DBPs (Procházka et al., 2019). These findings suggest that alterations to genes associated with immune and inflammatory pathways may play an important role in the adverse health effects of DBPs exposure (Procházka et al., 2019). An observational study conducted to evaluate short-term molecular changes after DBP exposure in swimmers reported changes in serum immune markers (Vlaanderen et al., 2017), blood metabolome (van Veldhoven et al., 2018), blood transcriptional patterns and microRNA (Espín-Pérez et al., 2018), and blood genotoxicity markers (Font-Ribera et al., 2019) after short-term DBP exposure in swimming pools. Nitrate is a precursor of nitric oxide in the body (Hezel et al., 2015), which is a free radical involved in carcinogenesis (Ying and Hofseth 2007) and plays a role in intestinal inflammation (Kubes and McCafferty 2000). Despite these pieces of evidence, there is only partial understanding of the modes of action of DBPs and nitrate, to provide biological plausibility to the associations observed with colorectal cancer risk. We conducted a study aimed to identify immune markers in blood linked both to colorectal cancer risk and exposure to nitrate and trihalomethanes in drinking water.

# 2. Methods

## 2.1. Study design, population and response rates

This study is nested in the Multi Case-Control project in Spain (MCC-Spain, www.mccspain.org), conducted from September 2007 to November 2013 in nine Spanish provinces (Castaño-Vinyals et al., 2015). Cases were histologically confirmed incident colorectal cancer patients [*International Statistical Classification of Diseases and Related Health Problems* (10th revision) (ICD-10): C18, C19, C20, D01.0, D01.1, D01.2]. Cases were identified through active searches including periodic visits to hospital departments (i.e., gastroenterology, oncology, general surgery, radiotherapy, and pathology), and were interviewed at the hospital as soon as possible after diagnosis (median of 58 days). Controls were frequency matched to cases by sex, age (±5 years), and area of residence. Controls were selected from the general population through the lists of randomly selected family practitioners in primary care centers sharing the catchment area of the participating hospital, and were interviewed at the primary health centre. Selection criteria included age (20–85 years old), residence in the hospital catchment area for ≥6 months before recruitment, and being able to answer the epidemiological questionnaire.

For the present analysis we selected a random subset of non-current smoking cases (43% never smokers, 57% former-smokers) with more complete long-term exposure assessment to THMs and nitrate in drinking water (≥70% years with known exposure), enrolled in 4 provinces (Barcelona, Cantabria, Gipuzkoa, León). Selected controls were never and ex-smokers individually matched to cases by area, age and sex. Average response rates ranged from 54% (Cantabria) to 80% (Barcelona, León) among cases, and from 58% (Barcelona, Gipuzkoa) to 68% (Leon) among controls. A total of 198 cases and 205 controls were included. The study protocol was approved by the ethics review boards of the participating centers, and all participants signed an informed consent before recruitment.

## 2.2. Individual information

Trained interviewers administered a questionnaire to ascertain personal information on sociodemographic factors; lifestyle (smoking, alcohol consumption, physical activity, etc.); anthropometrics (height, weight); residential history and type of water consumed in each residence (municipal, bottled, other); occupational history; medical history and pharmaceuticals use; and family history of cancer. Amount of water ingested was ascertained as number of glasses per day consumed on average as an adult, recorded separately at home, workplace and other places; as bottled, municipal, and other sources. Dietary habits were collected through a validated semiquantitative and self-administered food frequency questionnaire to collect average diet in the last year (Martin-Moreno et al., 1993). The World Cancer Research Fund/American Institute of Cancer Research (WCRF/AICR) score, incorporating six of the WCRF/AICR recommendations (regarding body fatness, physical activity, foods and drinks that promote weight gain, plant foods, animal foods, and alcoholic drinks) was used in our analysis, since it previously showed independently a significant association with colorectal cancer risk in the population where our study is nested (Romaguera et al., 2017). Detailed information on the operationalization of the score can be found in (Romaguera et al., 2017). Briefly, we assigned for each component, 1 point when the recommendation was met, 0.5 point when it was partially met and 0 point otherwise. When available, the quantitative criteria provided in the recommendations were used as cut-off points and intermediate cut-off points used in the literature or defined by the authors were used otherwise. For the recommendations including several sub-recommendations (foods and drinks that promote weight gain or plant foods), the final score was the average of each sub-recommendation score. Two recommendations (regarding the preservation, processing and preparation of foods and the recommendation on dietary supplements) and the two special recommendations (for cancer survivors and for mothers to breastfeed) were not included in the score. As the WCRF/AICR recommendations were not ranked according to priority, all major recommendations were summed to contribute equally to the total WCRF/AICR score. Therefore, the total WCRF/AICR score ranged from 0 to 6, with higher scores indicating greater concordance with the WCRF/AICR recommendations. The score was further categorized into sex-specific tertiles according to the distribution of the score in controls.

## 2.3. THM and nitrate exposure assessment

The concentration of THMs and nitrate in the public drinking water of the study area, as well as water source (% ground, surface source) and treatment history were ascertained through questionnaires to water utilities and local authorities. In addition, monitoring levels (2004–2010) were provided by the Sistema de Información Nacional en Aguas de Consumo (SINAC). Routine monitoring records from questionnaires and SINAC were used to estimate annual average concentration for the study municipalities back to 1940, covering 83% of person-years. For years when measurements were not available, the annual concentration was estimated based on available measurements in the municipality and historical information on main predictors (water source and treatment). Based on the assumption that concentrations remain approximately constant for a given water source and treatment, we imputed the municipal average THM and nitrate concentrations to the past, as long as source and treatment remained unchanged. When source changed, the percentage of ground vs. surface water was used as a weight to increase nitrate and reduce THM levels. Treatment changes were also considered to impute historical THM levels, applying a correction factor based on current measurements with information on treatment. Water samples were collected in a subset of study municipalities to measure THMs and nitrate, showing consistent geographical patterns relative to historical levels (Espejo-Herrera et al., 2013; Villanueva et al., 2012). Nitrate is expressed as $NO_3^-$.

The concentration in drinking water was merged by year and municipality of residence of study subjects to estimate personal exposure indices. Study subjects were assigned an average concentration of trihalomethanes (chloroform, bromodichloromethane, dibromochloromethane, bromoform) and nitrate based on the residences since age 18 years (referred as "long-term") and in the last 3 years before the interview (referred as "recent"), excluding the last 2 years in both cases (i.e. year 3 prior the interview). Exposure through ingestion was estimated using personal information on type of water consumed (tap, bottled, private well) and the amount (litres/day). Residential levels were assigned when subjects reported drinking tap water. When subjects reported bottled water consumption, a zero value of THM and 6.1 mg/L of nitrate was assigned, based on the literature (Espejo-Herrera et al., 2013; Font-Ribera et al., 2010). When subjects reported well water consumption, a value of 0.3, 0.3, 0.8, and 1.8 µg/l, respectively, were assigned for chloroforom, bromodichloromethane, dibromochloromethane, and bromoform, based on observations from other study areas with ground water and THM measurements. Current consumption of water from private wells mainly occurred in Leon region, were a well water sampling was conducted to measure nitrate. Levels from well water samples in León (range 0.5–93 mg/L) were assigned to well water consumers in this area. Missing values were assigned for well water in other areas. Details on the exposure assessment have been published elsewhere (Espejo-Herrera et al., 2016; Villanueva et al., 2017).

## 2.4. Blood samples and inflammation markers

Cases were interviewed as soon as possible after diagnosis (median of 58 days). Blood samples were collected after the interview by venipuncture, processed and stored at −80 °C. Previous to the laboratory analyses, serum samples were randomized by case-control status, area, sex and age, in order to minimize potential batch effects. The selected panel of immune markers was based on a previous study evaluating the link with DBP exposure among swimmers (Vlaanderen et al., 2017). We used two panels, a 4plex containing IL-8, EGF, and MPO and a 7plex containing IP-10, VEGF, IL-17, MDC, periostin, IL-1ra, G-CSF, and eotaxin. In total, 11 immune markers in serum were assessed using and R&D Systems (Abingdon, UK) Luminex® screening assay according to the protocol described by the manufacturer. In addition, C-reactive protein (CRP) was assessed using a R&D Systems Solid Phase Sandwich ELISA. Quality control samples were run in duplicate with the study samples in each batch. Concentration of IP-10, IL-1rA, periostin, and EGF was above the detection limit in 100% samples. Concentration of G-CSF were below the limits of quantification in >40% of the samples and was therefore excluded from main analyses. Imputation has been applied for inflammatory markers detected at least in 40% samples (eotaxin, VEGF, MDC, IL-8, IL-17E, MPO, and CRP), based on maximum likelihood estimation procedure (Lubin et al., 2004). To allow for plate to plate variation we imputed based on each plate-specific limit of quantification and included plate as a predictor variable in the imputation model. Median values of coefficient variation ranged from 2.3% (MPO) to 9.8% (eotaxin/CCL11).

## 2.5. Statistical analysis

We used density plots to present inflammation marker concentration distributions and we natural log transformed all markers, as distributions were right-skewed. We estimated the association between exposure and inflammation markers among controls using linear regression models. All regression analyses were adjusted for area, age, sex, education, smoking status (former vs non-smokers), WCRF score, and plate. We adjusted for multiple comparisons by calculating for each immune marker q-values based on p-values reported by the regression models using the Benjamini-Hochberg procedure. We defined q = 0.05 as the threshold for a significant association. We estimated the association between tertiles of inflammation markers (defined according controls)

and colorectal cancer risk using logistic regression adjusting for sex, age, education, smoking, area, WCRF score, and plate. We performed a mediation analysis to evaluate the role of protein levels (mediators) in the association between water contaminants (exposures) and colorectal cancer (outcome), focusing on exposures significantly associated with colorectal cancer risk. Indirect effects, direct effects and total effects were estimated to evaluate the effect of the mediators in the relation between the exposures and colorectal cancer (VanderWeele 2016).

## 3. Results

Study subjects were mostly men (62%), from Leon (47%), with primary school (44%), and slightly overweight, with average body mass index at 27 Kg/m$^2$ similarly among cases and controls (Table 1). Age of study participants ranged from 48 to 85 years, with a mean of 67 years (Table 1). Current smokers were not included, and prevalence of former smokers was higher among cases (57%) compared to controls (41%) (Table 1). The subset analysed was older, and with a higher proportion of cases, women, lower education, never smokers, high WCEF score adherence, and subjects from Leon compared to the complete set (Supplementary Material Table 1).

The distribution of the inflammation markers is shown in Fig. 1. Density plots of natural log transformed serum immune markers among cases and controls show slight visual differences for most proteins and minimal differences for eotaxin and IL-17. Correlation between immune markers is shown in Supplementary Table 2. C-reactive protein, IP-10 and periostin were uncorrelated with the rest of proteins. The highest correlation was between IL-17E and IL-8 (rho = 0.701). Correlations that were above 0.6 were IL17E-EGF (rho = 0.638), IL17E-IL1ra (rho = 0.613), IL17E-MPO (rho = 0.627). The distribution of proteins by TNM stage among cases did not show any pattern (Supplementary Table 3).

Fig. 2 shows the distribution of exposure variables among controls. Median concentrations of chloroform, brominated THMs and total THMs are, respectively, 11.6, 10.2, and 26.6 µg/L for the recent exposure window and 16.5, 9.0, and 23.51 µg/L for the long-term. Estimated ingested median levels of chloroform, brominated THMs and total THMs were, respectively, 2.1, 4.2, and 7.6 µg/day total THMs for the recent

exposure window and 4.4, 4.7, and 9.8 µg/L for the long-term. Median nitrate exposure was slightly higher for the recent exposure window compared to the long-term, with residential level of 3.4 mg/L vs. 3.0 mg/L, and ingestion exposure of 4.9 mg/day vs. 3.4 mg/day.

Correlations between exposure variables are shown in Supplementary Table 4. Highest correlations were found for THM and its components (CHCl3, brominated THMs), for a given exposure window (recent/long term) and route (residential levels/ingestion). For example, rho between recent THM-ingestion was 0.94 with BrTHM, and 0.978 with CHCl3. Recent and long-term exposures showed high correlation coefficients, ranging from rho = 0.70 (BrTHM ingested) to rho = 0.92 (nitrate ingested). Ingestion vs. residential levels for a given exposure window showed moderate correlations for nitrate (rho = 0.57 for recent exposure, rho = 0.60 for long-term exposure), and lower or no correlations for the THMs, CHCl3 and BrTHM.

The association between drinking water contaminants and colorectal cancer risk is shown in Fig. 3. Residential levels and ingestion exposure of brominated THMs were associated with an increased odds ratio of colorectal cancer, both for the recent and the long-term exposure. Recent nitrate exposure, either through ingestion or residential levels, were positively related to colorectal cancer risk. However, long-term nitrate exposure was not related with colorectal cancer risk. Residential chloroform levels were negatively related with colorectal cancer risk, particularly for the recent exposure. Associations for colon and rectal cancer sites separately are shown in Supplementary Fig. 1.

Table 2 shows the association between the recent exposure to water contaminants and serum concentration of immune markers among controls. The associations for chloroform and brominated THMs are mutually adjusted, as well as between nitrate and total THMs. Serum concentrations of EGF increased with residential levels of brominated THMs (16.8% for a µ10 g/l increase), chloroform (24.4%), and total THMs (17.3%), and also with ingestion of total THMs (6.8%). Serum concentrations of IL-8 increased with residential levels of brominated THMs (31.9%), chloroform (53.7%), and total THMs (34.8%), and also with ingestion of total THMs (14.5%). Serum concentrations of IL-17E increased with residential levels of chloroform (38.9%), and total THMs (20.3%), and also with ingestion of total THMs (10.8%). Serum concentrations of eotaxin increased with residential levels of chloroform (18.7%). Nitrate was unrelated to the serum immune markers. Associations for independent models for the 4 exposures are shown in Supplementary Table 5. In these models, EGF, IL-17E and IL-8 are associated with total THMs (residential and ingested levels), and ingested brominated THMs. Results for nitrate are similar to the mutually adjusted models (Table 2). Long-term exposure led to null associations with all serum immune markers (Supplementary Table 6). Generalised additive models show linear associations between EGF, IL-17E and IL-8 with recent residential brominated THM levels for the lower exposure range, reaching a plateau at approximately 30 µg/L (Supplemental Fig. 2A). Exposure-response between inflammation markers and total THM show a similar shape (Supplemental Fig. 2C), while a linear association between chloroform and EGF, IL-17E and IL-8 and is observed in all the exposure (Supplemental Fig. 2B).

Associations between tertiles of serum immune markers and colorectal cancer risk adjusted for area, sex, education, smoking, WRCF score, and plate are shown in Supplementary Table 7. A monotonic and significant increase in colorectal cancer is observed for VEGF, and periostin. A marginally significant positive association is observed for IL-1ra, and a monotonic negative trend is observed for EGF. Differences by cancer site were minimal. Results from the mediation analysis are shown in Supplementary Table 8. Periostin was identified as the only protein apparently mediating the effect of water contaminants on colorectal cancer risk, contributing 6.9% to the effect of recent ingested brominated THMs on colorectal cancer risk.

**Table 1**
Characteristics of study subjects (N = 403, 198 cases, 205 controls).

| | Control Mean (SD) | Case Mean (SD) | P value |
|---|---|---|---|
| Age, years | 66.8 (8.1) | 67.8 (9.4) | 0.278 |
| BMI, kg/m$^2$ | 27.2 (4.2) | 27.4 (4.6) | 0.536 |
| | N (%) | N (%) | |
| **Sex** | | | |
| Men | 124 (60.5) | 126 (63.6) | |
| Women | 81 (39.5) | 72 (36.4) | 0.515 |
| **Education** | | | |
| Less than primary school | 51 (24.9) | 56 (28.3) | |
| Primary school | 100 (48.8) | 78 (39.4) | |
| Secondary school | 33 (16.1) | 48 (24.2) | |
| University | 21 (10.2) | 16 (8.1) | 0.098 |
| **Smoking** | | | |
| Never | 121 (59) | 86 (43.4) | |
| Former | 84 (41) | 112 (56.6) | 0.002 |
| **World Cancer Research Fund (WCRF) score** | | | |
| Low adherence | 62 (30.2) | 59 (29.8) | |
| Medium adherence | 65 (31.7) | 68 (34.3) | |
| High adherence | 72 (35.1) | 54 (27.3) | |
| Missing | 6 (2.9) | 17 (8.6) | 0.049 |
| **Area** | | | |
| Barcelona | 51 (24.9) | 46 (23.2) | |
| Cantabria | 29 (14.1) | 29 (14.6) | |
| Gipuzkoa | 30 (14.6) | 30 (15.2) | |
| Leon | 95 (46.3) | 93 (47) | 0.984 |
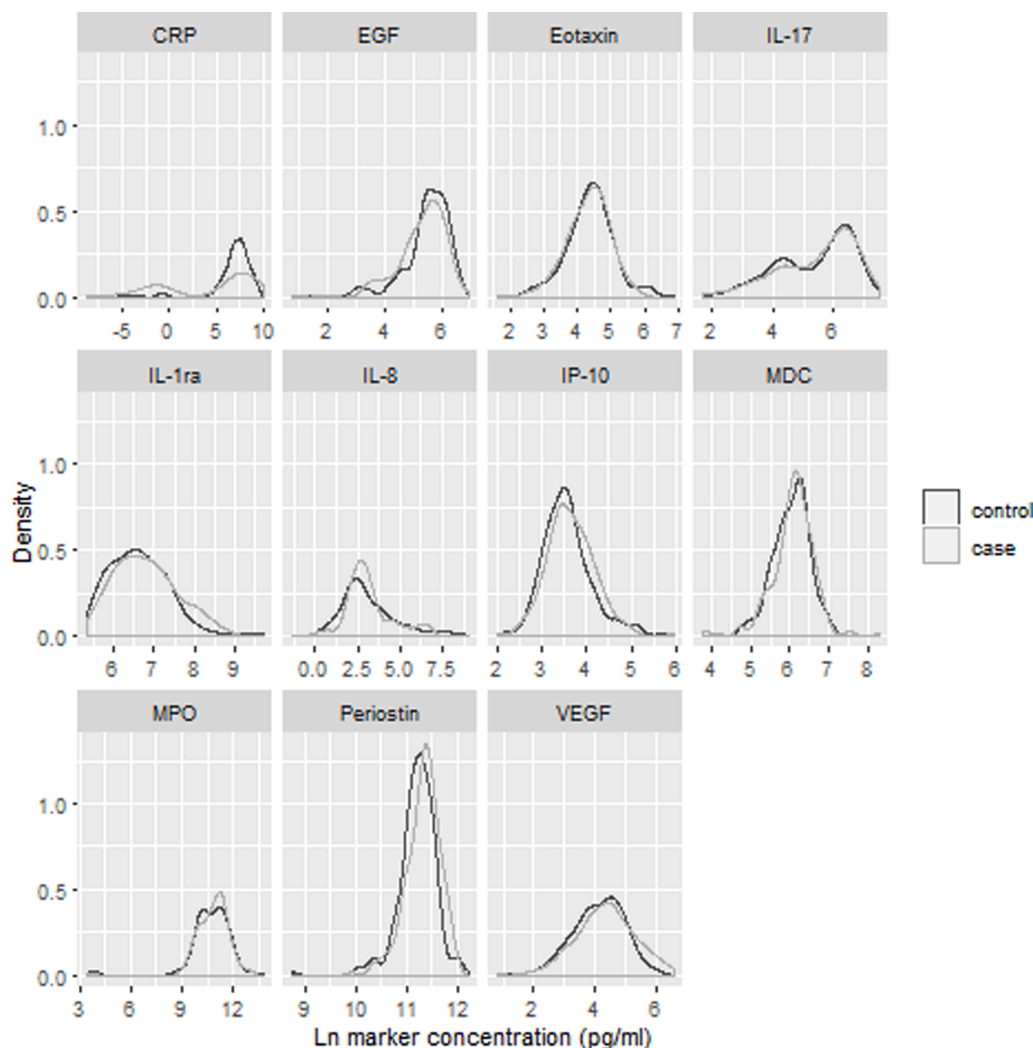
**Fig. 1.** Distribution of natural log transformed serum immune marker concentrations (pg/ml) among cases and controls.

## 4. Discussion

We examined a range of markers of exposure to trihalomethanes and nitrate in drinking water through different routes and time-windows in relation to colorectal cancer risk and blood inflammation markers. Higher associations were found for recent compared to long-term exposure. Residential levels, that reflect exposure through different exposure routes and pathways (e.g. inhalation, dermal contact, water and water-based food, etc.) showed higher associations compared to ingestion exposure markers, both for nitrate and trihalomethanes. An increased risk of colorectal cancer was associated with brominated THMs, total THMs, and nitrate, while a reduced risk was observed for chloroform, as previously reported in MCC-Spain. We observed that EGF, IL-17E and IL-8 in blood were positively associated with recent exposure to chloroform, brominated trihalomethanes, and total trihalomethanes among controls. Eotaxin was positively associated with chloroform levels. No associations with inflammation markers were found for nitrate. Although we identified proteins significantly associated with THM exposure, none of them were in the pathway between THM exposure and colorectal cancer risk.

### 4.1. Comparison with previous evidence

There is very limited human evidence on the link between inflammation markers in blood and exposure to THMs and nitrate in drinking water. A previous study in swimmers exposed to trihalomethanes, mainly through inhalation and dermal contact, evaluated a common set of immune markers (Vlaanderen et al., 2017): eotaxin (CCL11), CRP, EGF, G-CSF (below the quantification limit in our study in >40% samples), IP-10 (CXCL10), IL-17 (also known as IL-25), IL1-ra, IL-8, MPO, periostin, and VEGF. Vlaanderen et al. (2017) identified a decrease in IL-8, CCL11, CXCL10, and CRP, and an increase in IL-1ra among volunteers swimming during 40 min in a chlorinated pool. However, the study design did not allow to disentangle the effect attributable to short-term THM exposure from that attributable to physical activity and the exposures are not completely comparable between both studies. Evaluation of drinking water samples using *in vitro* assays have shown inflammatory responses through the nuclear factor Kappa N (NF-κB) GeneBLAzer assay (Hebert et al., 2018), a cell-based assay involving tumour necrosis factor alfa (TNFα) (König et al., 2017). However, the use of *in vitro* bioassays to evaluate drinking water quality is an emerging research area (Dingemans et al., 2019; Neale and Escher 2019), and there is still limited evidence available.

The association between drinking water contaminants and colorectal cancer risk in our study is consistent with the previously published results for the whole MCC study (Espejo-Herrera et al., 2016; Villanueva et al., 2017). The associations we identify at the observed exposure levels are consistent with previous studies conducted in different settings. Increased risks of colorectal cancer associated with nitrate levels at concentrations comparable to the present study have been observed in
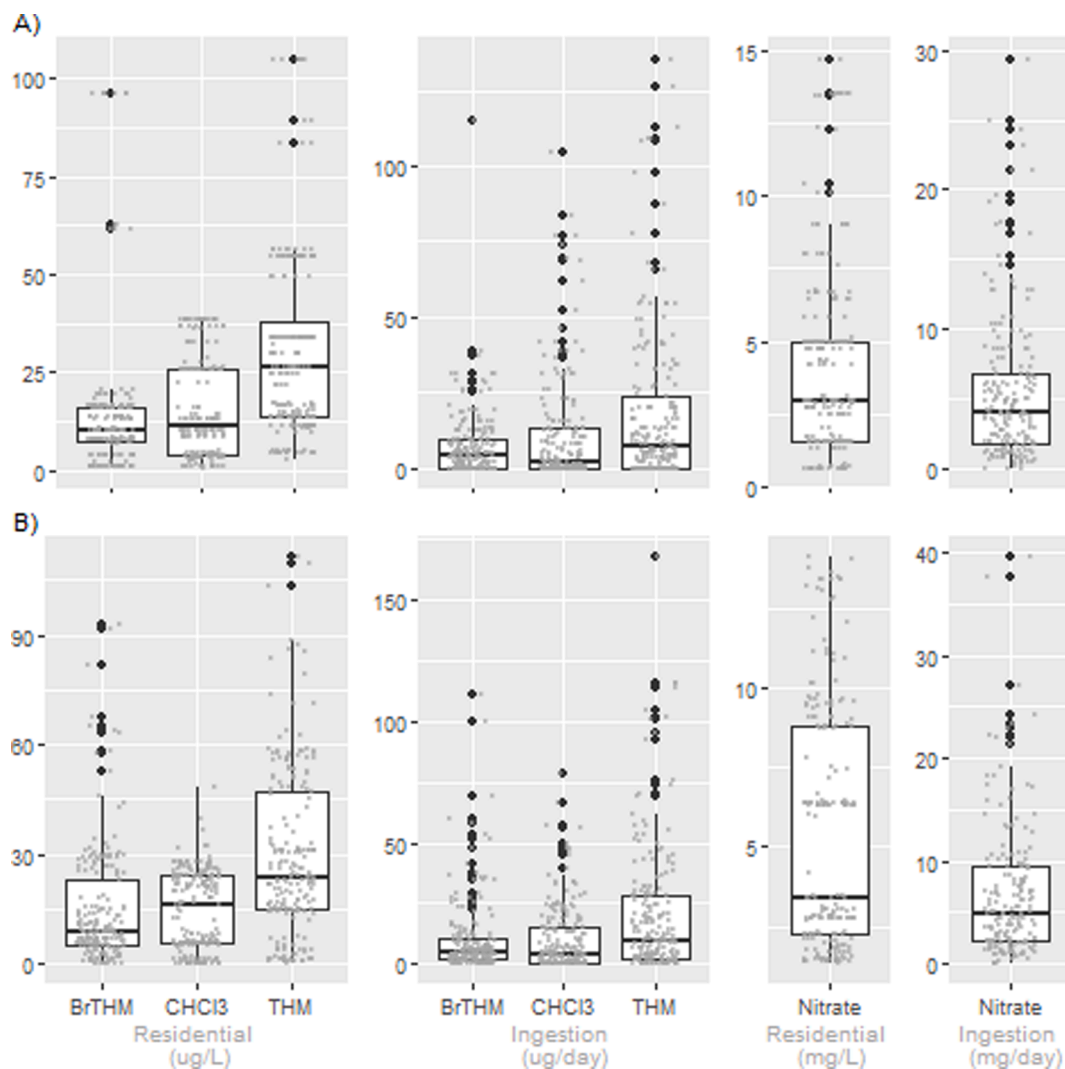
**Fig. 2.** Distribution of brominated THMs (BrTHM), chloroforom (CHCl3), total trihalomethanes (THMs), and nitrate exposure among controls, for recent period (A) and long-term (B).

a large cohort study conducted in Denmark (Schullehner et al., 2018). Similarly, Jones et al. (2019) detected an increased risk of rectal cancer associated with THM exposure at levels lower than the observed in our study (Jones et al., 2019).

### 4.2. Biological role of proteins related with colorectal cancer

In our study, concentration of VEGF, IP-10 and periostin in blood was positively related with colorectal cancer odds ratio with a monotonic dose–response. The vascular endothelial growth factor (VEGF) is involved in the formation of vessels in tumor tissue, and increased VEG receptor signaling has been found in CRC (Raskov et al., 2014). Together with EGF receptor, VEGR receptor antibodies have been proposed as therapeutic tools in CRC treatment (Mármol et al., 2017). The interferon gamma-induced protein 10 (IP-10), also known as C-X-C motif chemokine 10 (CXCL10) is involved in several biological functions such as inhibition of angiogenesis, antitumor activity, inhibition of bone marrow colony formation, chemoattractant for human monocytes and T cells, and promotes T cell adhesion to endothelial cells (Angiolillo et al., 1995). Periostin plays a wide variety of roles in tissue development along with the disease. Periostin overexpression has been reported in several types of cancer (González-González and Alonso 2018), and has been associated with the epithelial-mesenchymal transition in cancer (Hoersch and Andrade-Navarro 2010). In our study, results from a

mediation analysis showed that periostin partly explained the total effect between recent ingested brominated THMs and colorectal cancer risk and the moderate percentage of mediated effect. However, we cannot rule out chance in our observed mediation of periostin on colorectal cancer risk, given the multiple exposure-protein combinations evaluated. Unexpectedly, EGF levels in blood were negatively related to colorectal cancer odds ratio. The epidermal growth factor (EGF) is a soluble protein that stimulates epidermal proliferation, decreased apoptosis and enhanced tumor cell motility and neo-angiogenesis (Arteaga 2002). EGF is encoded by a polymorphic gene, and A61G polymorphism (rs4444903) has been linked with colorectal cancer risk (Zhu et al., 2019). EGF receptor is overexpressed in patients with colorectal cancer and it is has been proposed as a therapeutic target for CRC (Raskov et al., 2014). Although evidence suggest that the EGF receptor is involved in the pathogenesis and progression of different carcinoma types (Normanno et al., 2006), our results show a reduced odds ratio of colorectal cancer with EGF concentration in blood. The inconsistency may be partly explained by the different tissues considered in different studies (tumour in previous cancer studies vs. blood in our study).

### 4.3. Biological role of proteins related with trihalomethane exposure

EGF, IL-17E, and IL-8 in controls were consistently related to recent exposure to chloroforom, brominated THMs and total THMs. In
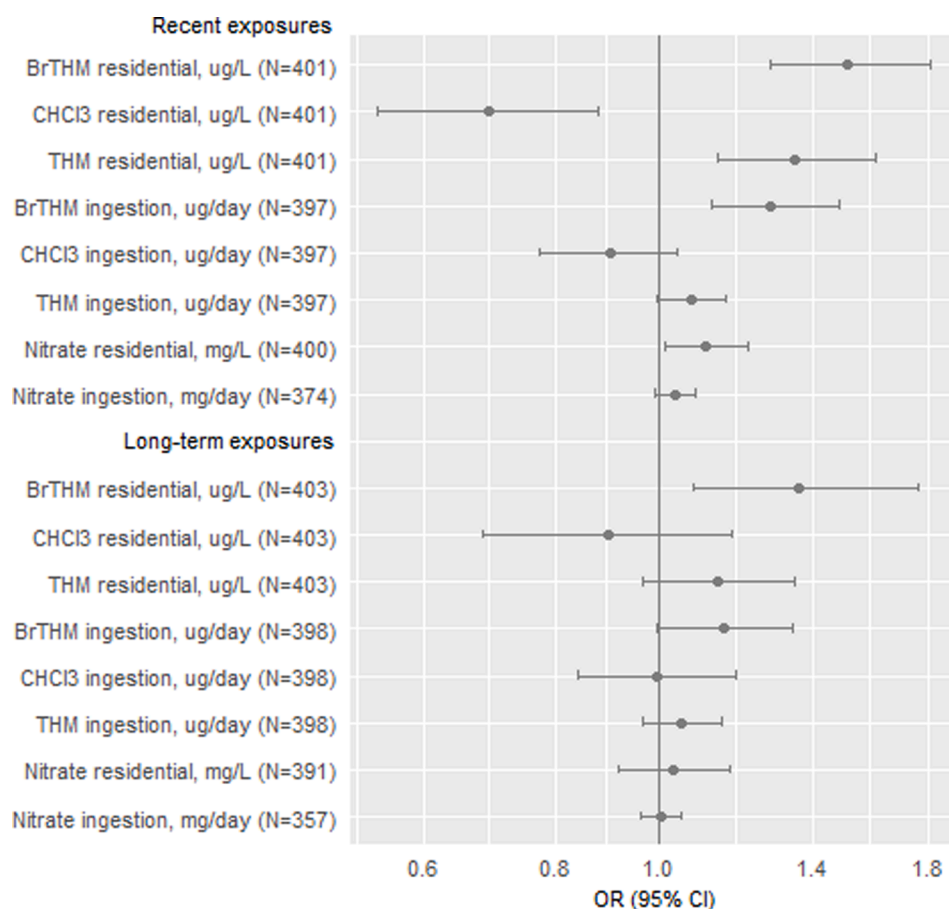
**Fig. 3.** Odds ratio (OR) and 95% confidence intervals (CI) of colorectal cancer associated with a 10 μg/L increase in brominated trihalomethanes (Br-THM), chloroform (CHCl3) and total trihalomethanes (THM), and 1 mg/L increase in nitrate in drinking water levels. Adjusted for area, age, sex, education, smoking, WCRF score, and plate.

addition, eotaxin was positively associated with chloroform. Interleukin 17-E (IL17E), also known as IL-25, is a proinflammatory cytokine produced by many cell types. Among other functions, IL17E stimulates the production of interleukin-8 (IL-8) and promotes the development of T-helper (Th) 2 immune response against several bowel infections (Lee et al., 2001; Owyang et al., 2006). IL17E plays a relevant role controlling immunity of the gut (Owyang et al., 2006), and has shown antitumor activity *in vivo* in several human cancers including melanoma, breast, lung, colon and pancreatic cancers (Benatar et al., 2010). On the other hand, IL-17 has been involved in metastasis and prognosis of colorectal cancer (Razi et al., 2019). Interleukin 8 (IL-8), also known as chemokine (C-X-C motif) ligand 8 - CXCL8, is a chemokine produced by macrophages and other cell types such as epithelial cells, airway smooth muscle cells and endothelial cells. IL-8 induces chemotaxis, causing target cells (e.g. neutrophils) to migrate to the site of infection, and stimulates phagocytosis. *In vitro* and *in vivo* studies with colon cancer cell lines have shown that overexpression of IL-8 promotes tumour growth, metastasis, chemoresistance and angiogenesis (Ning et al., 2011). The eotaxins are a subfamily of chemokines that specifically target eosinophils. Increased eotaxin-1 (CCL11) levels in blood plasma are associated with aging (Villeda et al., 2011). In addition to airway inflammation, eotaxin-1 is increasingly recognized as a major mediator of intestinal inflammation (Adar et al., 2014). Although our study requires replication, results suggest that THMs are involved in inflammation processes, which could be a mechanism of action leading to adverse health effects.

### 4.4. Exposure assessment

Hypochlorite was the main disinfectant used in all the study areas

and THMs is an appropriate DBP marker for the relevant exposure period. Exposure measurement error was a concern particularly for the long-term exposure windows. Uncertainty of exposure estimates for the recent period is lower than in the long-term, given that exposure is mostly assigned based on actual measurements, while the long-term exposure is largely based on imputations using predictors of the concentrations (water source, treatment). In addition, the number of years with THM exposure assigned increased from 68% in 1940–1950 to 99% in 2000–2010 in the study population, with an average of 95% for all the study years. Comparison of collected data through water companies with our own water measurements (Espejo-Herrera et al., 2013; Villanueva et al., 2012) showed comparable concentrations. Although we used strategies to minimise measurement error, such as include subjects with known exposure at least for 70% of the exposure window, the potentially lower exposure misclassification in the recent exposure window could partly explain the higher associations for the recent exposures compared to the long-term. In addition, the link with recent exposure would be biologically plausible given that immune changes are expected to act in late stages of carcinogenesis (Zamarron and Chen, 2011). Inability to account for exposure outside the home may have introduced error in the ingestion THM estimates, although most of total water was consumed at home (74%). Study participants showed a stable residential history, reporting a median number of 3 different residences. The current residence at the time of interview had a median duration of 37 years, and generally corresponded to the longest residence. Consequently, most of the study subjects were expected to live in the same residence 3 years before the interview and blood extraction. Finally, we cannot rule out uncontrolled confounding by other water contaminants although this is unlikely. Regulated chemicals other than THMs and

**Table 2**
Percentage change in the log-transformed protein concentration for a 10-unit increase in recent exposure to brominated THMs (BrTHMs), chloroform (CHCl3), and total trihalomethanes (THM) and 1-unit increase in nitrate exposure, from a linear regression adjusted for area, age, sex, education, smoking, WCRF score, and plate.

| | BrTHM Change (qvalue) | CHCl3 Change (qvalue) | THM Change (qvalue) | Nitrate Change (qvalue) |
|---|---|---|---|---|
| **Residential levels** | | | | |
| | µg/L N = 204 | µg/L N = 204 | µg/L N = 204 | mg/L N = 204 |
| CRP | −9.44% (0.504) | 9.47% (0.691) | −7.03% (0.594) | −4.05% (0.782) |
| EGF | **16.84% (0.017)** | **24.43% (0.042)** | **17.27% (0.017)** | 0.00% (0.999) |
| Eotaxin | 5.79% (0.226) | **18.65% (0.042)** | 6.92% (0.226) | −1.12% (0.782) |
| IL-17[E] | 18.06% (0.056) | **38.89% (0.042)** | **20.27% (0.032)** | −2.40% (0.782) |
| IL-1ra | 6.51% (0.226) | 8.83% (0.356) | 6.54% (0.236) | 0.26% (0.999) |
| IL-8 | **31.94% (0.043)** | **53.73% (0.042)** | **34.79% (0.028)** | −3.15% (0.782) |
| IP-10 | −1.64% (0.611) | −6.51% (0.356) | −2.21% (0.553) | 0.80% (0.782) |
| MDC | 4.64% (0.226) | 3.35% (0.522) | 4.25% (0.236) | 0.82% (0.782) |
| MPO | 12.13% (0.226) | 32.11% (0.070) | 14.09% (0.226) | −2.15% (0.782) |
| Periostin | 2.49% (0.348) | 4.74% (0.356) | 2.24% (0.422) | 0.90% (0.782) |
| VEGF | 8.25% (0.226) | 9.78% (0.356) | 7.67% (0.236) | 1.60% (0.782) |
| **Ingestion** | | | | |
| | µg/day N = 201 | µg/day N = 201 | µg/day N = 189 | mg/day N = 189 |
| CRP | 1.14% (0.950) | 6.15% (0.732) | 3.90% (0.700) | 1.17% (0.885) |
| EGF | 14.36% (0.218) | 3.75% (0.732) | **6.83% (0.042)** | 0.34% (0.885) |
| Eotaxin | −0.69% (0.950) | 3.51% (0.732) | 1.60% (0.582) | 0.34% (0.885) |
| IL-17[E] | 15.58% (0.400) | 8.78% (0.732) | **10.76% (0.042)** | −0.50% (0.885) |
| IL-1ra | 6.97% (0.571) | 3.58% (0.732) | 4.16% (0.225) | 0.31% (0.885) |
| IL-8 | 39.29% (0.178) | 5.07% (0.732) | **14.47% (0.042)** | 2.03% (0.885) |
| IP-10 | −1.06% (0.950) | 1.68% (0.732) | 0.66% (0.700) | −0.13% (0.892) |
| MDC | 0.73% (0.950) | −0.98% (0.734) | −0.61% (0.700) | 0.32% (0.885) |
| MPO | −1.95% (0.950) | −4.15% (0.732) | −3.44% (0.582) | −2.97% (0.885) |
| Periostin | 4.11% (0.520) | −0.05% (0.979) | 1.25% (0.550) | 0.60% (0.885) |
| VEGF | 4.11% (0.950) | 4.58% (0.732) | 3.77% (0.367) | 2.36% (0.885) |

*Note:* Brominated THMs and chloroform are mutually adjusted, as well as total THMs and nitrate.

nitrate showed levels around the quantification limit in the study areas (Espejo-Herrera et al., 2013) and are not likely to be relevant confounders in the context of colorectal cancer.

## 5. Strengths and limitations

This is the first human observational study aiming to identify inflammatory markers linked to long-term exposure to widespread drinking water contaminants, in order to elucidate biological mechanism underlying epidemiological associations. However, the partial coverage of immune markers may lead to undetected immunological responses that could be relevant (Procházka et al., 2019). We focused our selection of immune markers towards the exposure rather than towards the outcome, and we failed to include circulating levels of inflammatory cytokines that could be relevant in the aetiology of colorectal cancer such as IL-6 (Kakourou et al., 2015). Although models were adjusted by the main known risk factors of colorectal cancer, residual confounding by unaccounted variables (e.g. specific dietary

pattern varying by area) cannot be ruled out. Although response rates were moderate to low and varied between areas, there was no trend with exposure levels among areas. In addition, reasons for participating (or not) were likely independent on the hypotheses evaluated here (drinking water quality). Thus, eventual selection bias from low response rates is expected to affect minimally the results. Although the study design is valid to identify inflammatory markers linked to the examined exposures (among controls), the case-control design and the analysis of post-diagnostic samples hampers the ability to identify etiologically relevant associations with colorectal cancer. Measurement of protein markers after diagnosis constitute a limitation, given that protein changes may occur after cancer diagnosis. Exposure measurement error remains a concern given the retrospective nature of the study, and particularly for the long-term exposure window. This error has likely affected the precision of point exposure estimates, but less likely affected exposure rankings, which may have reduced the ability to detect significant associations. Sample size was limited for stratified analysis by sex or cancer site (colon, rectum). However, exposure-cancer associations in the main population did not show effect modification by sex and cancer site (Espejo-Herrera et al., 2016; Villanueva et al., 2017).

## 6. Conclusions

Our results suggest that trihalomethane exposure is involved in inflammation processes. The lack of association between nitrate exposure and inflammation markers suggest other biological mechanisms would be involved in the link between this exposure and colorectal cancer. Further studies are warranted to elucidate potential carcinogenic mechanisms of disinfection by-products and nitrate in drinking water. Replication in prospective longitudinal cohorts and the use of a wider set of inflammatory markers is warranted.

## CRediT authorship contribution statement

**Cristina M. Villanueva:** Conceptualization, Methodology, Writing – original draft, Supervision. **Ana Espinosa:** Formal analysis, Visualization, Data curation. **Esther Gracia-Lavedan:** Methodology. **Jelle Vlaanderen:** Investigation, Methodology, Writing – review & editing. **Roel Vermeulen:** Conceptualization, Supervision. **Antonio José Molina:** Investigation. **Pilar Amiano:** Investigation. **Inés Gómez-Acebo:** Investigation. **Gemma Castaño-Vinyals:** Investigation, Project administration. **Paolo Vineis:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **Manolis Kogevinas:** Conceptualization, Supervision, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106873.

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