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Exposure to disinfection by-products in swimming pools and biomarkers of genotoxicity and respiratory damage – The PISCINA2 Study

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ABSTRACT

Background: Swimming in pools is a healthy activity that entails exposure to disinfection by-products (DBPs), some of which are irritant and genotoxic.

Objectives: We evaluated exposure to DBPs during swimming in a chlorinated pool and the association with short-term changes in genotoxicity and lung epithelium permeability biomarkers.

Methods: Non-smoker adults (N = 116) swimming 40 min in an indoor pool were included. We measured a range of biomarkers before and at different times after swimming: trihalomethanes (THMs) in exhaled breath (5 min), trichloroacetic acid (TCAA) in urine (30 min), micronuclei in lymphocytes (1 h), serum club cell protein (CC16) (1 h), urine mutagenicity (2 h) and micronuclei in reticulocytes (4 days in a subset, N = 19). Several DBPs in water and trichloramine in air were measured, and physical activity was extensively assessed. We estimated interactions with polymorphisms in genes related to DBP metabolism.

Results: Median level of chloroform, brominated and total THMs in water was 37.3, 9.5 and 48.5, µg/L, respectively, and trichloramine in air was 472.6 µg/m³. Median exhaled chloroform, brominated and total THMs increased after swimming by 10.9, 2.6 and 13.4, µg/m³, respectively. Creatinine-adjusted urinary TCAA increased by 3.1 µmol/mol. Micronuclei in

Abbreviations: BMI, body mass index; Br-THMs, brominated THMs; CYP2E1, cytochrome P450 2E1 gene; CC16, club cell protein; CC16, club cell protein gene; DBP, disinfection by-product; GSTT1, glutathione S-transferase theta 1 gene; GSTZ1, glutathione S-transferase zeta 1 gene; HAA, haloacetic acid; IQR, interquartile range; MHR, maximum heart rate; MN, micronuclei; MN-PBL, micronuclei in peripheral blood lymphocytes; MN-Ret, micronuclei in reticulocytes; SNP, single-nucleotide polymorphism; TCAA, trichloroacetic acid; THM, trihalomethane; TTHMs, total trihalomethanes

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lymphocytes and reticulocytes, urine mutagenicity and serum CC16 levels remained unchanged after swimming. Spearman correlation coefficients showed no association between DBP exposure and micronuclei in lymphocytes, urine mutagenicity and CC16. Moderate associations were observed for micronuclei in reticulocytes and DBP exposure.

Conclusions: The unchanged levels of the short-term effect biomarkers after swimming and null associations with personal estimates of exposure to DBPs suggest no measurable effect on genotoxicity in lymphocytes, urine mutagenicity and lung epithelium permeability at the observed exposure levels. The moderate associations with micronuclei in reticulocytes require cautious interpretation given the reduced sample size.

1. Introduction

Swimming is a healthy and highly practiced sport, but swimming in pools entails a considerable exposure to disinfection by-products (DBPs). Hundreds of DBPs have been identified in swimming pool water (Richardson et al., 2010), and some of them are genotoxic and carcinogenic in animals (Richardson et al., 2007). The major exposure route for volatile DBPs such as trihalomethanes (THMs) is inhalation (Erdinger et al., 2004; Marco et al., 2015), whereas dermal absorption, accidental ingestion or inhalation of aerosol may be more relevant for other DBPs (Cardador and Gallego, 2011). Exposure to THMs through drinking water and showering has been consistently associated with bladder cancer risk (Villanueva et al., 2004). A study also suggested an increased risk of bladder cancer with pool attendance (Villanueva et al., 2007). In addition, indoor swimming pools accumulate volatile DBPs in the air, including irritant compounds such as trichloramine. Several studies have described a higher prevalence of respiratory disorders such as asthma among pool workers and professional swimmers (Villanueva and Font-Ribera, 2012).

In order to provide evidence on the mechanisms of action of DBP exposure in swimming pools, we previously conducted an experimental study (PISCINA study) in 50 healthy adults swimming during 40 min in an indoor chlorinated swimming pool (Kogevinas et al., 2010; Font-Ribera et al., 2010). Individual DBP exposure was measured as the THM concentration in exhaled breath after swimming. Several genotoxicity and respiratory biomarkers were assessed in biosamples collected before and at different times after swimming, including micronuclei (MN) and comet assay in peripheral blood lymphocytes, urine mutagenicity, MN in exfoliated urothelial cells in urine, exhaled nitric oxide, cytokines and 8-isoprostane in exhaled breath condensate, and surfactant protein D and Club cell protein (CC16) in serum. The individual exposure to bromoform, one of the most genotoxic THMs (Richardson et al., 2007) measured as exhaled levels, was related to an increase in MN frequency in peripheral blood lymphocytes (MN-PBL), urine

mutagenicity (Kogevinas et al., 2010) and serum CC16 (Font-Ribera et al., 2010). Several genes involved in DBP metabolism have been shown to modify bladder cancer risk (Cantor et al., 2010) and were also evaluated in the PISCINA study (Kogevinas et al., 2010).

The PISCINA study was the first to assess DBP genotoxicity among volunteers swimming in a chlorinated pool and other similar studies have not been conducted. The assessment of short-term changes in respiratory biomarkers after swimming has been more studied, especially for lung epithelium permeability, with inconsistent results (Carbonnelle et al., 2002; Carbonnelle et al., 2008; Nordberg et al., 2012; Llana-Belloch et al., 2015). The role of physical activity is a key aspect in this type of studies measuring biomarkers before and after swimming, which has not been properly tackled in previous studies.

We aimed to assess short-term changes in biomarkers of genotoxicity and lung epithelium permeability in relation to DBP exposure in swimming pools in a new study (PISCINA2) to replicate the previous PISCINA study in an independent and larger population. We used the effect biomarkers that were previously related to DBP exposure (serum CC16 for lung epithelium permeability and urine mutagenicity and MN-PBL for genotoxicity) and genotyped twelve polymorphisms in three genes involved in DBP metabolism. In addition, in this study we measured an extensive range of DBPs in water, air and biofluids, and used several indicators of physical activity. We finally also evaluated MN in reticulocytes (MN-Ret), which is a newer and potentially more sensitive genotoxicity biomarker (Kissling et al., 2007).

2. Methods

2.1. Study design

The PISCINA2 study included currently non-smoker and non-professional swimmers between 18 and 40 years old, recruited through open advertisements at local research centres, universities, social

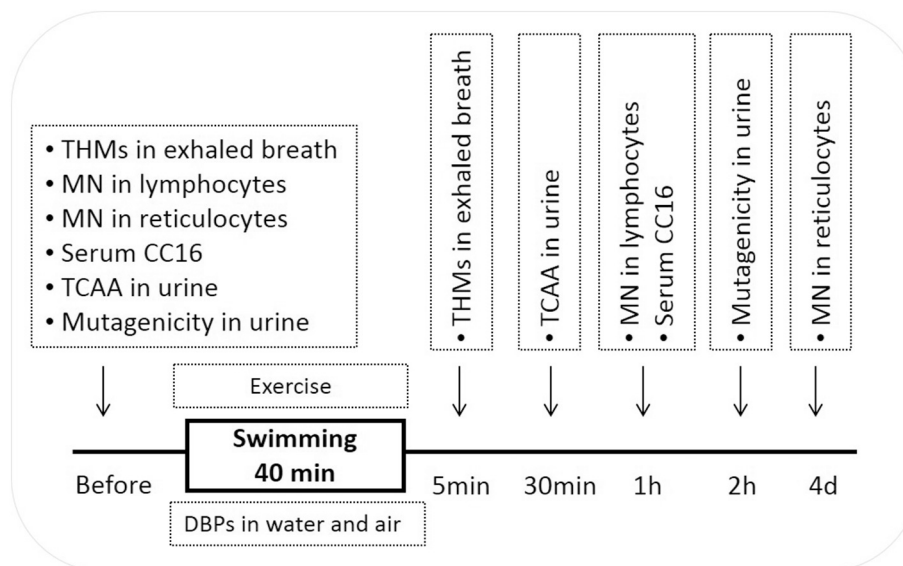


Fig. 1. Study design and timing of sample collection.

THMs: trihalomethanes; TCAA: trichloroacetic acid; MN: micronuclei; CC16: Club cell protein. DBPs: disinfection by-products.

media, and personal contacts. Participants were asked to avoid swimming pool attendance the week before, exercise three days before and up to three days after swimming (until the last sample collection), and showering in the morning of the study, and were provided with DBP-free bottled water during the study period. In total, 116 volunteers were recruited over 30 experimental days (June–December 2013), who were asked to swim during a fixed time of 40 min in a single indoor chlorinated pool in Barcelona. Four participants were evaluated per day, with start swimming times ranging from 10:00 a.m. to 11:42 a.m. Participants were asked to swim at a free pace, resting as much as wanted. Before and after swimming, a battery of measurements and biological samples were collected to evaluate exposure and effect biomarkers according to a strict schedule (Fig. 1). Timing for biosample collection was tailored to best capture the aimed biomarkers considering the specific pharmacokinetics, and was based on previous evidence (Kogevinas et al., 2010; Font-Ribera et al., 2010; Cardador and Gallego, 2011; Abramsson-Zetterberg et al., 2000). Blood samples were obtained by venipuncture. Biological samples and measurements at baseline (before swimming) and within 30 min after swimming (exhaled THMs, urine sample) were obtained in a room inside the sports centre but separated from the swimming pool area. Afterwards, participants were taken by taxi to the Clinical Trial Unit in the Research Centre in a 10 min drive to collect the remaining samples, including blood samples at 1 h, 24 h and 4 days after swimming and urine samples at 2 h after swimming (Fig. 1). The study was approved by the ethics committee of the research centre following international regulations, and all volunteers signed an informed consent before participation.

2.2. Effect biomarkers

2.2.1. Micronuclei in peripheral blood lymphocytes (MN-PBL)

Four millilitre of blood were obtained with heparinized vacutainers before and 1 h after swimming. To determine the frequency of binucleated cells with MN and the total number of MN, PBLs were cultivated using cytochalasin-B. Slides were stained with 4'-6-diamino-2-phenylindol (DAPI) a fluorescent marker with strong affinity for DNA sequences (Fenech, 2007; Thomas et al., 2009). Each slide was dyed with 15 μ L of DAPI, at a final concentration of 1 μ g/mL, plus Antifade (for preservation of the fluorescent signal), and scored immediately or few days after dying at \times 100 with a fluorescent microscope. Samples were stored in the dark at 4 °C. Five hundred binucleated cells with well-preserved cytoplasm were scored in two replicates by two trained scorers (total of 1000 cells per sample) and an average of the two scores was counted per sample. As a quality control, each scorer evaluated blindly six times a slide and in all instances except one, differences within scorer was not higher than one binucleated MN. Differences between scorers for the 230 samples evaluated were as follows: 21.7% (n = 50) had identical score between the two scorers; 58.3% (n = 134) differed by one or two binucleated MN; 12.2% (n = 28) differed by 3; 7.8% (n = 18) differed by 4 or more binucleated MN. These 18 slides with differences above 3 binucleated MN between scorers were cross-counted and it was found that differences were due to the replica per se and not to differences in criteria between scorers.

2.2.2. Micronuclei in reticulocytes (MN-Ret)

Two millilitre of blood was obtained with heparinized vacutainers before and four days after swimming. Samples were stored at room temperature until processed within the collection day. Immunomagnetic isolation was done to retain transferrine positive immature reticulocytes from blood (Abramsson-Zetterberg et al., 2000). These immature reticulocytes are the very youngest erythrocytes, recently extruded from the bone marrow into the peripheral blood system. After fixation, samples were stored at 21 °C for three to four days and then at 4 °C. MN frequency was counted automatically by flow-cytometry in all the isolated cells per sample. The assay was performed in the last half of the study sample (n = 54). From the 107 samples collected, 56 (52%) had detectable levels of isolated cells, with a mean of 27,251 cells/sample

(SD = 25,258). Only 19 participants had isolated reticulocytes for the before and after swimming sample and could be used for the analysis.

2.2.3. Urine mutagenicity

Urine samples (30 mL) were collected before and 2 h after swimming. Mutagenicity was evaluated with the *Salmonella* (Ames) mutagenicity plate-incorporation assay in strain YG1024 with S9 mix. The mutagenic potencies of samples, expressed as revertants per millilitre-equivalent (rev/mL-eq), were calculated from the slope of the regression over the linear portion of the dose–response curves.

2.2.4. Serum CC16

Four millilitre of blood were collected before and 1 h after swimming and centrifuged at 2500 rpm for 15 min at 4 °C. 100 μ L of serum was aliquoted and stored at –80 °C. CC16 was analyzed by ELISA using commercial kits (Biovendor Laboratorní medicína a.s., Brno, the Czech Republic). Intra- and inter-assay coefficients of variation were 3.19% and 5.63% respectively. The minimum detectable concentration in serum was set at 46 pg/mL.

2.3. Biomarkers of exposure

2.3.1. Trihalomethanes in exhaled breath

Trihalomethanes (THMs) including chloroform (CHCl₃), bromodichloromethane (BDCM), dibromochloromethane (DBCM), and bromoform (CHBr₃) were measured in exhaled breath before and right after swimming. The volunteers breathed deeply and blew three times into the Bio-VOC™ sampler (150 mL), retaining the air for 10 s between each inspiration and expiration. The alveolar air was collected in a last additional breath/blow cycle. A screw-in plunger was used to steadily discharge the sample contained in the Bio-VOC™ into a sorbent tube trap. These stainless steel tubes were 3.5" (89 mm) long and 1/4" o.d. They were packed with 200 mg sorbent Tenax TA 35/60 mesh (Markes International Ltd., UK) which was preconditioned using helium of 5 N grade at 100 mL/min at 320 °C for 2 h and then at 335 °C for 30 min. In posterior conditioning cycles they were reconditioned at 335 °C for 20 min and the same flow carrier gas. One cleaned, the tubes were capped with brass storage caps fitted with PTFE ferrules and were stored at 4 °C in a clean environmental free-solvent atmosphere.

The THMs retained in the tubes were then desorbed and concentrated in a thermal desorption unit equipped with a Unity Series 2 Thermal Desorber and an Ultra 50:50 Multi-tube Auto-sampler (Markes International Ltd.). They were desorbed at 300 °C for 5 min and the vapors were concentrated into a cold trap at –20 °C at 40 mL/min carrier flow (Helium 5 N grade) without split. The cold trap, packed with a graphitized carbon sorbent for General Purpose by Markes International Ltd., was rapidly heated to 300 °C for 5 min with a 7.5 mL/min flow of helium and the THMs were transferred to the gas chromatograph (GC) with a split flow of 6 mL/min. Only 20% of the samples in the sorbent tube was introduced into the GC column by heating at 200 °C. The THMs were finally measured in a Gas Chromatograph 7890 (Agilent Technologies) equipped with a DB-5MS UI capillary column (60 m \times 0.32 mm, 1 m; Agilent J & W GC Columns) at 1.5 mL/ml of carrier gas flow (Helium, 5 N grade). The GC oven temperature program was hold for 10 min at 40 °C; 5 °C/min to 150 °C; hold for 10 min; 15 °C/min to 210 °C; and finally hold for 10 min. This instrument was coupled to a mass spectrometer (5975C Inert XL MSD) with a source in electronic impact mode (Agilent Technologies). The GC–MS heat transfer line was heated at 280 °C. The MS source and MS quadrupole temperatures were 230 °C and 150 °C, respectively. The quantitative determination was carried out in selected ion monitoring Mode. Dwell time was set at 50 ms for each ion scanned. THMs concentrations were expressed as micrograms per cubic meter (μ g/m³).

2.3.2. Trichloroacetic acid (TCAA) in urine

Ten millilitre of urine were collected before and 30 min after swimming, stored at –20 °C and shipped in dry ice to the laboratory. TCAA

concentrations were measured using solid phase extraction followed by liquid chromatography tandem mass spectrometry (LC-MS-MS). Creatinine in urine was also measured in order to control for excretion rate, and TCAA concentration was expressed as creatinine-adjusted levels ($\mu\text{mol TCAA/mol creatinine}$). Details on sampling collection and analysis have been published previously (Font-Ribera et al., 2016).

2.4. Environmental measurements

Chlorine, chloramines, pH, and four THMs were measured in water for each of the participants. Nine haloacetic acids (HAAs) in water samples were measured once per experiment day. Water samples were composite samples collected at two different points of the pool while participants were swimming, stored at 4 °C until laboratory analyses. Quenching agents in water samples were used for THMs ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ and KHSO_4) and HAAs (NH_4Cl) measurements. Air samples to measure trichloramine were collected once per day using a pump with a constant sampling flow rate (1.2 L/min for a mean \pm SD of 115 ± 32 min) within 1 m from the water and at a height of 60 cm above the floor level. The instrumental analyses were performed at the Institute for Risk Assessment Sciences at Utrecht University (Utrecht, The Netherlands), following the method described by Hery et al. (1995). Details on sample collection and chemical analysis have been previously described (Font-Ribera et al., 2016).

2.5. Physical activity and personal information

A study field worker sitting in front of the pool counted the distance swam and the time swimming by each participant with a counter and a chronometer. Energy expenditure (in kcal) was estimated using the swimming speed and the weight of the participant, assuming that swimming at 46 m/min equals 8.3 metabolic equivalent tasks (METs; kcal per kg per hour) (Ainsworth et al., 2011). Subjective exertion and shortness of breath were evaluated before and after swimming using the Borg scale (Borg, 1970) with the following questions “In a scale from 0 to 10, how would you describe your exertion/shortness of breath?”, and the change after swimming was calculated for both parameters. Heart rate during the 40 min was measured using a heart rate monitor (Polar RCX5, Polar Electro Oy, Kempele, Finland), and the intensity of physical activity was calculated in relation to the individual theoretical maximum heart rate (MHR) (Gulati et al., 2010). The heart rate < 50%, between 50 and 69%, and > 69% of MHR were considered as low, moderate, and high intensity, respectively. Weight and height were measured with standard procedures and the body mass index (BMI) was calculated. Questionnaires were used to collect information on sociodemographic data, health status, lifestyle, frequency of swimming pool attendance and other physical activities.

2.6. Gene selection and genotyping

Blood was collected in EDTA tubes and stored at -80 °C until DNA extraction. DNA was extracted using QIAamp DNA Blood Mini Kit (QIAGEN) and was measured with NANODROP 8000 (Thermo-Scientific) and all samples were diluted to a fixed concentration of 2.5 ng/mL. Twelve polymorphisms in three genes involved in DBP metabolism were genotyped: three single-nucleotide polymorphisms (SNPs) in the glutathione S-transferase zeta 1 (*GSTZ1*) gene, eight SNPs in the Cytochrome P450 2E1 (*CYP2E1*) gene by Taqman allele discrimination assay (Life Technologies) and a common deletion in the glutathione S-transferase theta 1 (*GSTT1*) gene. For a complete list of SNPs see Appendix Table A2. For quality control purposes, duplicate samples (5% of the total numbers of samples) were repeated for each SNP, and no template controls (NTCs) were included in each plate. All polymorphisms could be successfully analyzed in the 116 participants (the quality control of genotypes was assured with > 99% of concordance). All polymorphisms were also in Hardy-Weinberg

equilibrium and minor allele frequencies were similar to those described in the International HapMap Project for European individuals (International HapMap Consortium, 2003).

2.7. Statistical analysis

Mean or median values were reported to describe central tendencies. 9%, 3%, 19% and 4% of exhaled breath samples before swimming were below the detection limit for chloroform ($0.108 \mu\text{g}/\text{m}^3$), bromodichloromethane ($0.009 \mu\text{g}/\text{m}^3$), dibromochloromethane ($0.004 \mu\text{g}/\text{m}^3$) and bromoform ($0.007 \mu\text{g}/\text{m}^3$), respectively, and were imputed half the detection limit value. The concentrations of bromodichloromethane, dibromochloromethane and bromoform were summed up and reported as brominated THMs (Br-THMs). Differences between measures before and after swimming were tested using Wilcoxon signed-rank test. The change in the level of exposure and effect biomarkers after swimming was calculated (concentration after–before and mutagenic potency (slope) after–before in the case of urine mutagenicity). The distribution of short-term effect biomarkers was evaluated with a test for normality evaluating skewness and kurtosis. MN-PBL change showed a normal distribution while MN-Ret, urine mutagenicity and CC16 change showed skewed distributions. Spearman correlation was used to estimate the association between the change in the effect biomarkers with exposure to DBPs and exercise. Potential confounding was evaluated among individual covariables including age, sex, body mass index, current swimming practice, distance swam, time swimming, energy expenditure, intensity of exercise, exertion, and shortness of breath. Confounders were identified empirically as those covariables related both with the effect biomarker and exposure. Only distance swam was identified, associated with CC16 and DBP exposure. Spearman partial correlation adjusting for distance swam was used for CC16, based on robust linear regression. 95% confidence intervals of correlation coefficients were estimated through bootstrap with 1000 iterations. The p-value threshold for statistical significance was set up at < 0.05 . Genetic analyses were performed assuming a dominant genetic model, considering the most frequent allele as a reference category. To evaluate interactions between changes in THMs and gene variants, we included an interaction term for dichotomous genotype and exhaled THMs in linear regression models. All statistical analyses were performed with the statistical package STATA 12.0 (StataCorp. College Station, TX, USA).

3. Results

A total of 116 subjects (56 men, 60 women) participated in the study, with a median age of 23.9 years (Table 1). The majority was university students and 28% attended swimming pools at least once a month. The median distance swam during the study session was 1.1 km and median percentage time at high intensity physical activity was 71%, according to heart rate parameters. There was an increase in the exertion and shortness of breath scores after swimming (3 and 2 points, respectively). Energy expenditure was unrelated to age, while it was higher for men than for women (Appendix Table A1). BMI was negatively related to distance swam and positively to energy expenditure and shortness of breath.

Median concentrations of DBPs in pool water were 37.3 $\mu\text{g}/\text{L}$ for chloroform, 0.7 $\mu\text{g}/\text{L}$ for bromoform, 48.5 $\mu\text{g}/\text{L}$ for total THMs (TTHMs), and 111.2 $\mu\text{g}/\text{L}$ for total HAAs (Table 2). Median level of trichloramine in air was 472.6 $\mu\text{g}/\text{m}^3$, ranging from 248.5 to 858.3 $\mu\text{g}/\text{m}^3$. Additional information on DBP levels in water and air is shown in Table 2. For technical reasons some of the samples were lost and the final observations are shown in Table 2.

There was a significant increase after swimming in the level of all exposure biomarkers (Table 3). Median increase in exhaled breath after swimming was 10.9 $\mu\text{g}/\text{m}^3$ for chloroform (range 2.1, 35.1), 2.6 $\mu\text{g}/\text{m}^3$ for Br-THMs (range 0.1, 8.5) and 13.4 $\mu\text{g}/\text{m}^3$ for TTHMs (range 2.6, 42.6). Creatinine-adjusted TCAA in urine increased a median value of

Table 1
Characteristics of the study population and the exercise performed (N = 116).

Variable	Median	Percentile 25, 75
Age, years	23.9	21.3, 28.9
Body mass index, kg/m ²	23.0	21.3, 25.5
Distance swam, km	1.1	0.8, 1.2
Time swimming, min	35.3	29.9, 38.7
Energy expenditure, kcal	204.5	166.9, 254.6
Percentage of time at high intensity (> 69% MHR)	70.6	33.1, 88.2
Exertion ^a	3	2, 4
Shortness of breath ^a	2	1, 3.5

MHR: maximum heart rate.

^a Difference in the Borg scale (0–10 points), measured before and after swimming.

3.1 µmol/mol (range -9.23, 38.89), and 15 subjects showed higher levels before compared to after swimming. None of the effect biomarkers showed a significant change after swimming (Table 3).

The association between individual characteristics and change in biomarkers are shown in Table 4. Men showed a larger increase in exhaled Br-THMs after swimming, while women had a larger increase in creatinine-adjusted TCAA in urine. Several measures of physical activity were positively related to the increase in THMs in exhaled breath after swimming, while distance swam and energy expenditure were negatively related to the increase in creatinine adjusted TCAA in urine. The change in the effect biomarkers did not differ by personal characteristics, except for serum CC16, whose change was negatively correlated to BMI and positively correlated to distance swam (Table 4). The baseline level of serum CC16 was negatively correlated with BMI and significant differences were also detected by regular physical activity and the polymorphism in the *CC16* gene (Appendix Table A2). The baseline frequency of MN-PBL was positively correlated to age and was higher among ever smokers. Urine mutagenicity before swimming differed among different genotypes of polymorphisms in *CYP2E1* and *GSTZ1* (Appendix Table A2). The frequencies of MN-PBL and MN-Ret were positively but not significantly correlated at baseline and after swimming (Spearman correlations of 0.38 (p-value 0.134) and 0.24 (p-value 0.330), respectively).

Table 5 shows the association between the change in short-term effect biomarkers and exposure. No associations were found for MN-PBL, urine mutagenicity and CC16. A moderate correlation was found between MN-Ret and DBP exposure, positive for chloroform (0.50), brominated THMs (0.55) and total THMs (0.56), and negative for urine TCAA (-0.59) (Table 5).

We examined interactions between Br-THMs and TTHMs and polymorphisms in *GSTT1*, *GSTZ1*, and *CYP2E1* metabolism genes in relation to frequency of MN-PBL (Appendix Table A3). Statistically significant interactions were observed between five SNPs in the *CYP2E1* gene, exposure to THMs and MN-PBL frequency; interactions for two SNPs (rs2249695 and rs2515641) remained statistically significant even after Bonferroni correction for multiple comparisons. There was no difference in the association between THM exposure and MN-PBL frequency, between the null *GSTT1* genotype (-/-; a deletion in both copies of the gene) and those with one (+/-) or none (+/+) of the copies deleted. There was also no interaction for three SNPs of the *GSTZ1* gene. We also examined the rs3741240 polymorphism of the *CC16* gene in relation to levels of the CC16 protein and found no interaction with levels of CC16 protein.

4. Discussion

We evaluated DBP exposure and markers of genotoxicity and lung epithelium permeability in 116 young subjects after swimming for 40 min in an indoor chlorinated pool. Personal exposure to disinfection by-products measured as exhaled chloroform, bromodichloromethane,

dibromochloromethane and bromoform, and urinary trichloroacetic acid increased after swimming. Among the four evaluated biomarkers of short-term effect (MN-PBL, urine mutagenicity, serum CC16, and MN-Ret in a subset of 19), only change in MN-Ret showed a moderate association with DBP exposure. However, sample size was small and interpretation of these finding should be cautious.

MN-PBL is a well-established biomarker of DNA damage and in adults has been associated with cancer risk in large longitudinal studies (Bonassi et al., 2011). It has also been linked to environmental exposures, both at long-term, including Br-THMs in tap water (Stayner et al., 2014), and at short-term, such as ozone (Holland et al., 2015). MN-PBL was determined by manually counting MN in 1000 binucleated cells per sample. MN-Ret has been recently proposed as a genotoxicity biomarker. It is more sensitive (Kissling et al., 2007), using an automated flow cytometer-based analysis, allowing the measurement of a large number of cells (i.e., around 27,000 per sample in the present study). In this method, no cultivation of cells occurs but a disadvantage is the complex sample preparation needed in order to isolate reticulocytes that occur in a very low proportion in blood (Abramsson-Zetterberg et al., 2000). MN in PBL and in reticulocytes reflects different exposures windows, as the half-life of the cells differs. A correlation between MN-Ret and MN-PBL frequencies has been found in previous studies both at baseline levels (Costa et al., 2011) and in the increase after an acute exposure (Stopper et al., 2005). The rather small numbers and differential influence of exposures other than those related to swimming, particularly for MN-Ret, complicate the direct interpretation of the associations. In the present study, positive correlations were found among the two assays but these were not statistically significant, probably due to the low sample size.

The present study has been conducted exactly in the same swimming pool of our previous PISCINA study (Kogevinas et al., 2010; Font-Ribera et al., 2010). The protocols of the two studies were very similar

Table 2
Level of disinfection by-products and related parameters in the swimming pool water.

Parameter	N	Median	Percentile 25, 75	Min	Max
Trihalomethanes (THMs) (µg/L)					
Chloroform	108	37.3	32.9, 43.8	24.0	61.6
Bromodichloromethane	108	7.1	5.4, 8.6	3.8	12.9
Dibromochloromethane	108	2.0	1.6, 3.0	0.9	4.7
Bromoform	108	0.7	0.5, 1.3	0.2	1.9
Brominated THMs	108	9.5	7.9, 12.6	5.7	19.4
Total THMs	108	48.5	43.6, 54.7	30.7	74.7
Haloacetic acids (HAAs) (µg/L)					
Dichloroacetic	30	29.4	22.3, 37.7	15.4	51.7
Trichloroacetic	30	62.9	57.4, 70.2	39.2	83.4
Bromochloroacetic	30	4.9	4.2, 6.2	2.4	8.8
Dibromoacetic	30	1.0	1.0, 1.8	0.5	3.1
Dichlorobromoacetic	30	11.8	9.4, 15.4	4.8	23.4
Total HAAs	30	111.2	95.5, 129.3	73.3	144.3
Chlorine (mg/L as Cl₂)					
Free chlorine	114	1.3	1.0, 1.6	0.6	2.0
Total chlorine	114	2.2	1.9, 2.3	1.6	2.7
Chloramines (mg/L as Cl₂)					
Monochloramine	114	0.2	0.1, 0.4	0.0	0.7
Dichloramine	112	0.3	0.0, 0.4	0.0	0.7
Trichloramine	114	< DL	< DL, 0.6	< DL	1.6
Other parameters					
Trichloramine in air (µg/m ³)	26	472.6	381.4, 533.5	248.5	858.3
pH	115	7.40	7.3, 7.6	7.1	8.0
Water temperature (°C)	115	28.0	27.8, 28.2	27.1	28.6
Air temperature (°C)	115	28.5	27.8, 29	26.5	29.5
Number of swimmers in the pool	115	23.0	16.3, 34.3	9.0	120.5

All samples were below the detection limit (DL) for chloroacetic acid (< 3 µg/L), bromoacetic acid (< 2 µg/L), dibromochloroacetic acid (< 2 µg/L), and tribromoacetic acid (< 5 µg/L).

Table 3

Levels of the biomarkers of exposure to disinfection by-products and genotoxicity, mutagenicity and lung epithelium permeability.

Biomarker	N	Before swimming Median (pctile25, 75)	After swimming Median (pctile25, 75)	Change Median (pctile25, 75)	p-Value ^a	Time after swim
DBP exposure						
Chloroform in exhaled breath, $\mu\text{g}/\text{m}^3$	116	0.4 (0.2, 0.6)	11.5 (8.0, 14.7)	10.9 (7.6, 14.3)	< 0.001	5 min
Br-THMs in exhaled breath, $\mu\text{g}/\text{m}^3$	116	0.1 (0.0, 0.1)	2.7 (2.0, 4.1)	2.6 (1.9, 3.9)	< 0.001	5 min
TTHMs in exhaled breath, $\mu\text{g}/\text{m}^3$	116	0.5 (0.3, 0.7)	14.4 (10.0, 18.4)	13.4 (9.7, 17.7)	< 0.001	5 min
TCAA in urine ^b , $\mu\text{mol}/\text{mol}$	113	2.5 (1.5, 4.8)	5.8 (3.2, 11.2)	3.1 (1.1, 6.1)	< 0.001	30 min
Effect						
MN-PBL (%)	113	4 (3,6)	4 (3,7)	0 (-2, 2)	0.185	1 h
MN-Ret (%)	19	0.9 (0.6, 1.3)	1.1 (0.8, 2.3)	0.4 (-0.4, 0.8)	0.277	4 days
Urine mutagenicity (rev/mL-eq)	88	1.3 (0.4, 2.0)	1.1 (0.4, 1.8)	-0.1 (-0.8, 0.5)	0.434	2 h
Serum CC16 (ng/mL)	105	11.1 (8.7, 13.4)	10.5 (8.2, 13.2)	-0.5 (-1.2, 1.2)	0.289	1 h

Br-THMs: brominated trihalomethanes; TTHMs: total trihalomethanes; TCAA: trichloroacetic acid. MN-PBL: micronuclei in peripheral blood lymphocytes. MN-Ret: micronuclei in reticulocytes.

^a Wilcoxon test.

^b Creatinine adjusted.

in study design, population characteristics, timing of sample collection and analytical techniques. Although median level of total THMs are similar in both studies, 45.5 $\mu\text{g}/\text{L}$ in PISCINA (Font-Ribera et al., 2010) compared to 48.5 $\mu\text{g}/\text{L}$ in the present study, the composition has completely changed. Brominated THMs were the most predominant THMs in the previous study, while chloroform is the predominant THM in the present. Median chloroform level was 16.1 $\mu\text{g}/\text{L}$ in the previous study (35% of total THMs) and 37.3 $\mu\text{g}/\text{L}$ in the present (77% of total THMs). This difference is also observed in exhaled levels, and although total THMs in exhaled breath in the present study is 14.4 compared to 7.9 $\mu\text{g}/\text{m}^3$ in the previous study, the levels of brominated THMs in exhaled breath have declined (from 0.5 to 0.1 $\mu\text{g}/\text{m}^3$ in exhaled bromoform levels). This decrease reflects mostly changes in the tap water

quality in the city (ASPB, 2012). Given that chloroform is a non-genotoxic THM, this could explain the lack of consistent effects on the genotoxicity and mutagenicity biomarkers in the present study, while the previous PISCINA study showed associations between MN-PBLs and urine mutagenicity with exhaled Br-THMs levels, that were not found for chloroform (Kogevinas et al., 2010). The results for MN-Ret require replication with a larger sample size. In particular, the association between MN-Ret and chloroform is difficult to understand since chloroform itself is not considered genotoxic, although chloroform could be an indicator of other DBPs. However, chance cannot be ruled out given the limited sample size.

As shown in Table 4, there are significant correlations between THM concentrations in exhaled breath and distance swam or energy

Table 4

Spearman correlation coefficients between individual characteristics and the changes in the exposure and effect biomarkers, and median values by sex and current swimming.

Covariables	Exposure biomarkers				Effect biomarkers			
	Chloroform in exhaled breath ($\mu\text{g}/\text{m}^3$)	Br-THMs in exhaled breath ($\mu\text{g}/\text{m}^3$)	TTHMs in exhaled breath ($\mu\text{g}/\text{m}^3$)	Creatinine adjusted TCAA in urine ($\mu\text{mol}/\text{mol}$)	MN-PBLs (%)	MN-Ret (%)	Urine mutagenicity (rev/mL-eq)	CC16 in serum (ng/mL)
N	116	116	116	113	113	19	88	105
Age	-0.08	0.04	-0.05	0.13	-0.12	-0.14	-0.01	-0.14
Body mass index	0.06	0.07	0.07	-0.05	0.07	-0.13	-0.03	-0.25*
Distance swam, km	0.26**	0.21*	0.25**	-0.32**	-0.06	-0.01	-0.18	0.25*
Time swimming, min	0.05	0.14	0.07	-0.21*	-0.01	-0.23	-0.11	0.11
Energy expenditure, kcal	0.27**	0.24**	0.27**	-0.33**	0.00	0.21	-0.14	0.19
High intensity ^a	0.16	0.21*	0.19*	-0.14	-0.06	0.02	-0.06	0.18
Exertion ^b	0.22*	0.37**	0.26**	0.03	-0.06	0.23	-0.09	0.01
Shortness of breath ^b	0.21*	0.34**	0.24**	0.03	0.04	0.37	0.02	-0.11
Sex								
Males, median	11.4	3.0*	15.0	1.8**	1	0.44	-0.11	0.01
Females, median	10.7	2.5*	12.7	4.0**	0	-0.47	-0.11	-0.66
Current swimming^c								
Yes, median	13.5**	3.2	16.7*	2.0	0	0.44	-0.12	-0.52
No, median	10.6**	2.6	13.0*	3.6	0.5	0.05	-0.11	-0.45

Br-THMs: brominated trihalomethanes; TTHMs: total trihalomethanes; TCAA: trichloroacetic acid. MN-PBL: micronuclei in peripheral blood lymphocytes. MN-Ret: micronuclei in reticulocytes.

p-Values from the Spearman correlations, except for sex and current swimming (Kolmogorov-Smirnov test).

* p-Value < 0.05.

** p-Value < 0.01.

^a > 69% maximum heart rate.

^b Difference in the Borg scale (0–10 points), measured before and after swimming.

^c At least once per month.

Table 5

Spearman correlation coefficients (95% confidence interval) between the change in short-term effect biomarkers and exposure to disinfection by-products and exercise.

Exposure	MN-PBL (%)	N	MN-Ret (%)	N	Urine mutagenicity (rev/mL-eq)	N	CC16 ^a (ng/mL)	N
Increase in exhaled breath								
Chloroform, ($\mu\text{g}/\text{m}^3$)	-0.16 (-0.35, 0.04)	113	0.50 (0.07, 0.93)	19	-0.13 (-0.33, 0.08)	88	-0.01 (-0.20, 0.18)	105
Brominated THMs, ($\mu\text{g}/\text{m}^3$)	-0.05 (-0.25, 0.14)	113	0.55 (0.13, 0.97)	19	-0.14 (-0.36, 0.07)	88	-0.05 (-0.25, 0.14)	105
Total THMs, ($\mu\text{g}/\text{m}^3$)	-0.15 (-0.34, 0.05)	113	0.56 (0.14, 0.97)	19	-0.14 (-0.36, 0.07)	88	-0.01 (-0.21, 0.18)	105
Increase in TCAA in urine, ($\mu\text{mol}/\text{mol}$)	-0.02 (-0.21, 0.17)	110	-0.59 (-0.94, -0.24)	18	-0.06 (-0.27, 0.16)	86	-0.09 (-0.30, 0.12)	102
Trichloramine in air, ($\mu\text{g}/\text{m}^3$)	0.05 (-0.14, 0.24)	99	0.20 (-0.37, 0.77)	17	-0.002 (-0.23, 0.23)	77	0.03 (-0.18, 0.25)	91
Distance swam, km	-0.06 (-0.25, 0.14)	113	-0.01 (-0.47, 0.45)	19	-0.18 (-0.39, 0.04)	88		
Energy expenditure, kcal	0.0003 (-0.19, 0.18)	113	0.21 (-0.25, 0.67)	19	-0.14 (-0.37, 0.09)	88		
High intensity (> 69% MHR)	0.06 (-0.25, 0.12)	109	0.92 (-0.45, 0.50)	19	-0.06 (-0.29, 0.17)	85		

MHR: maximum heart rate; MN-PBL: micronuclei in peripheral blood lymphocytes; MN-Ret: micronuclei in reticulocytes; TCAA: trichloroacetic acid.

^a Spearman's partial correlation coefficient adjusted for distance swam.

expenditure, $p < 0.05$ or 0.01 . These observations are consistent with inhalation as primary mechanism for THM incorporation into swimmers (Marco et al., 2015). However, the air-water partition mechanism of the pulmonary system leads to higher retention of the THM with lower Henry's Law volatility constants, e.g. those with higher degree of bromination (Marco et al., 2015) and therefore a selective relative enrichment of these compounds in the exhaled breath. Given that the exposure event occurred in a short time frame (10:00 to 11:42) in all volunteers, circadian variation of activity in genes involved in the metabolism of DBPs (Gängler et al., 2018) are expected to be minimal.

Short-term changes in serum CC16 may reflect an increase in lung epithelium permeability (Broeckaert et al., 2000), and it has been used as a sensitive biomarker to detect respiratory effects after short-term exposures such as air pollution (Stockfelt et al., 2012; Provost et al., 2014). The PISCINA2 study is the first to evaluate the relationship between the change in serum CC16 after swimming and the level of trichloramine in air, the main potential airway irritant in swimming pools. A large variability in trichloramine levels was observed during the thirty experimental days (from 248.5 to $858.3 \mu\text{g}/\text{m}^3$), with a median level comparable or even higher than in previous studies (Carbonnelle et al., 2002; Carbonnelle et al., 2008; Font-Ribera et al., 2010; Nordberg et al., 2012). The lack of association detected between trichloramine in air and serum CC16 suggests that DBP exposure in a single swimming session at the observed levels is not enough to affect this respiratory biomarker.

In the previous PISCINA study we detected a 3% increase in serum CC16 1 h after swimming (Font-Ribera et al., 2010), that was associated with DBPs exposure measured as THMs in exhaled air, and to physical exercise assessed through self-reported distance swam. This suggested a potential confounding effect by physical activity. In the current study we improved the measurement of physical activity by including different objective and subjective indicators. The distance swam, counted by a technician, was the measure that correlated best to changes in serum CC16 and also to the DBP exposure biomarkers. A previous before-after study detected a 44% and 52% increase in CC16 among 14 trained swimmers after swimming 45 min over a distance of 1500 m in a chlorinated and in a non-chlorinated swimming pool, respectively Carbonnelle et al. (2002). However, they did not find change in CC16 among 13 adults under a recreational regime during 2 h (1 h standing on the pool side, 1 h swimming freely). The lack of increase in serum CC16 despite a similar exercise compared to the previous study (median distance swam 1.1 km here vs. 0.95 km in PISCINA1) and the better

adjustment for physical activity may suggest that the intensity of physical activity was not sufficient to trigger this change.

We found interactions between some polymorphisms in the *CYP2E1* gene, exposure to brominated THMs and MN-PBL. Metabolism of DBPs is mediated by enzymes from the glutathione S-transferase (GST) and cytochrome P450 (CYP) families. Polymorphisms in *GSTT1*, *GSTZ1* and *CYP2E1* have been suggested to modify DBP-associated bladder cancer risk in a case-control study (Cantor et al., 2010) and to affect the DBP internal dose after swimming (Font-Ribera et al., 2016). Findings on gene-environment interactions that remained significant after correcting for multiple comparisons add to the global evidence on the importance of genetic polymorphisms in mediating the effect of disinfection by-products in water. Still the study had limited power to evaluate gene-environment interactions and we cannot exclude the possibility of having spurious findings.

The timing of the collection of biological samples is crucial when evaluating biomarkers of effect. For PBL-MN, CC16 and urine genotoxicity we followed the same timing as in our previous PISCINA study, where changes in those biomarkers were observed (Font-Ribera et al., 2010; Kogevinas et al., 2010). For other biomarkers (MN in reticulocytes, urinary TCAA) we considered previous knowledge and expert judgement of researchers to establish the times to collect specific samples to measure specific biomarkers.

The main advantage of the pre-post design is that individuals are compared with themselves, which is crucial for assessing small changes in biomarkers that may have a large inter-individual variability. A potential limitation is that external factors, such as physical activity, may still affect results even after adjustment. Alternative designs have been proposed such as swimming pools that use different disinfectants (Carbonnelle et al., 2002; Fernández-Luna et al., 2012; Llana-Belloch et al., 2015) or repeat the measurements after physical exercise without DBP exposure, such as indoor cycling or running. DBPs are, however, present in all disinfected pools but in a different composition and in our pool there was considerable variability in DBPs levels in different days. Other sports may not be comparable concerning biological changes due to physical activity. In this context, the PISCINA2 study followed a pre-post design with an accurate individual measurement of DBPs and physical activity, and the different factors were analyzed in multivariate models. A proper measurement of physical activity is especially relevant in the assessment of respiratory effects, but may be less crucial when assessing genotoxicity biomarkers.

We focused the exposure assessment on exhaled THMs, urinary TCAA and trichloramine in air. However, urinary TCAA has not been

validated as an exposure marker in short-term exposure settings like before-after swimming, which may partly explain the negative associations with Ret-MN. Although we measured other DBPs in water (Font-Ribera et al., 2016), we focused our main analysis in the biomarker-based exposure measures that provide more accurate personal exposure. Alternative analyses using DBPs in water as exposure did not show different results from the ones presented here. Concerning CC16, despite the focus of our study was on trichloramine, other DBPs present in air could contribute to lung irritation (e.g. dichloroacetonitrile, cyanogen chloride).

This is the largest study on the topic with 116 subjects evaluated. The exposure assessment to DBPs is also the most complete, with different classes of DBPs measured every day including not only THMs, but more carcinogenic DBPs such as HAAs, haloacetonitriles or nitrosamines (Plewa et al., 2008), that have been previously published (Font-Ribera et al., 2016). TCAA in urine was used to complement exhaled THMs as an exposure biomarker, although it was poorly correlated to other DBPs in water (Font-Ribera et al., 2016). Details on the levels, correlations and determinants of the evaluated exposure biomarkers have been described elsewhere (Font-Ribera et al., 2016). Timing of sample collection was based on previous studies (Abramsson-Zetterberg et al., 2000; Kogevinas et al., 2010; Font-Ribera et al., 2010; Cardador and Gallego, 2011), although evidence on the expression dynamics of the biomarkers after an environmental exposure is scarce. The DBP levels in the study swimming pool are similar to those reported in other studies (Chowdhury et al., 2014), supporting the external validity of these results. Another novelty of the PISCINA2 study is that we measured for the first time the effect of swimming in several omic signatures including proteomics (Vlaanderen et al., 2017),

metabolomics (van Veldhoven et al., 2018) and transcriptomics (Espín-Pérez et al., 2017) reported separately.

5. Conclusions

The unchanged levels of the short-term effect biomarkers after swimming and null associations with personal estimates of exposure to disinfection by-products suggest no measurable effect on genotoxicity in lymphocytes, urine mutagenicity and lung epithelium permeability at the observed exposure levels. The moderate associations with micronuclei in reticulocytes require cautious interpretation given the limited sample size.

Declaration of Competing Interest

Authors have no competing interests.

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Appendix A

Appendix Table A1

Distribution of physical activity indicators and associations with sex, age and body mass index.

Variable	All	Men	Women	Age	Body mass index
	Median (ptile 25, 75)	Median (ptile 25, 75)	Median (ptile 25, 75)	Spearman correlation coefficient	Spearman correlation coefficient
Distance swam, km	1.0 (0.8, 1.2)	1.1 (0.8, 1.2)	1.0 (0.9, 1.2)	−0.11	−0.20*
Energy expenditure, kcal	204.5 (166.9, 254.6)	228.8 (175.5, 310.2)*	197.5 (161.4, 233.8)*	−0.02	0.26**
Time swimming, min	35.3 (29.8, 38.7)	35.3 (27.9, 38.9)	35.3 (31.4, 37.9)	−0.07	−0.13
High intensity (> 69% MHR), time percentage	70.7 (33.1, 88.2)	63.6 (35.7, 84.4)	78.3 (33.1, 89.2)	−0.14	−0.10
Exertion, Borg scale (0–10)					
Before	1 (0, 2)	1 (0, 1.5)	1 (0, 2)	0.04	0.04
After	4 (3, 5)	4.5 (4, 6)*	3.7 (3, 5)*	−0.09	0.16
Difference	3 (2, 4)	3 (2.5, 5)	3 (2, 4)	−0.11	0.11
Shortness of breath, Borg scale (0–10)					
Before	0 (0, 1)	0 (0, 1)	0 (0, 1.5)	−0.15	−0.10
After	3 (1, 4)	2.5 (1, 5)	3 (1.5, 4)	−0.04	0.14
Difference	2 (1, 3.5)	2 (0, 4)	2 (1, 3)	0.00	0.21*

MHR: maximum heart rate. P-values from Kolmogorov-Smirnov test for sex and from Spearman correlations for age and body mass index.

* p-Value < 0.05.

** p-Value < 0.01.

Appendix Table A2

Potential determinants of the baseline levels of the genotoxicity and respiratory biomarkers.

Variable	Category	MN-PBL		MN-Ret		Urine mutagenicity		Serum CC16	
		N	Spearman's corr	N	Spearman's corr	N	Spearman's corr	N	Spearman's corr
Age (years)		113	0.34**	19	−0.05	97	0.00	108	0.08
Body mass index (kg/m ²)		113	−0.07	19	0.05	97	0.01	108	−0.22*

(continued on next page)

Appendix Table A2 (continued)

Variable	Category	MN-PBL		MN-Ret		Urine mutagenicity		Serum CCl6	
		N	Spearman's corr	N	Spearman's corr	N	Spearman's corr	N	Spearman's corr
		N	Median (pc25, pc75)	N	Median (pc25, pc75)	N	Median (pc25, pc75)	N	Median (pc25, pc75)
Sex	Male	56	4 (3, 5)	13	0.9 (0.7, 1.1)	47	1.2 (0.4, 2.1)	51	11.5 (9.1, 14.0)
	Female	60	5 (3, 7)	6	1.2 (0.6, 1.6)	50	1.0 (0.3, 1.6)	57	10.7 (7.9, 12.0)
Ever smoker	Yes	16	5 (4, 6.5) [*]	4	0.6 (0.4, 0.8)	16	0.9 (0.1, 1.4)	16	11.3 (9.2, 13.9)
	No	96	4 (3, 6) [*]	15	1.1 (0.8, 1.4)	81	1.3 (0.4, 1.9)	91	11.0 (8.2, 13.4)
Drinking water type	Bottled	52	5 (3, 6)	6	0.9 (0.6, 1.1)	45	1.0 (0.3, 1.9)	49	11.1 (8.7, 12.7)
	Tap filtered	21	4 (2, 8)	5	1.3 (0.9, 1.6)	15	1.3 (0.3, 1.5)	21	10.6 (9.2, 13.6)
	Tap unfiltered	32	4 (3, 6)	8	0.9 (0.6, 1.2)	30	1.1 (0.2, 1.5)	30	11.0 (7.4, 13.8)
Current swimming ^a	Yes	33	4 (3, 6)	9	1.1 (0.9, 1.3)	29	1.1 (0.1, 1.7)	32	11.0 (7.7, 13.5)
	No	80	5 (3, 6)	10	0.8 (0.5, 1.1)	68	1.1 (0.4, 1.9)	76	11.1 (8.7, 13.2)
Physical activity ^b	Low	53	5 (3, 6)	7	0.7 (0.3, 1.1)	49	1.2 (0.3, 1.7)	50	11.0 (9.2, 12.9)
	Medium	34	4.5 (2, 6)	5	1.0 (0.8, 1.3)	27	1.1 (0.2, 2.1)	33	9.1 (7.6, 12.4)
	High	25	4 (3, 6)	7	1.1 (0.6, 1.4)	20	1.1 (0.4, 1.7)	24	12.8 (10.8, 15.2) [*]
Asthma	Yes	11	4 (2, 5)	3	0.6 (0.5, 1.1)	10	0.4 (0.3, 1.0)	9	9.3 (8.8, 10.5)
	No	101	5 (3, 6)	16	1.0 (0.8, 1.3)	87	1.3 (0.4, 1.9)	98	11.1 (8.6, 13.6)
GSTT1 (glutathione S-transferase theta 1 gene)									
	Null	23	5 (2, 6)	2	1.6 (1.4, 1.8)	21	1.5 (1.0, 1.8)		
	Positive	90	4 (3, 6)	17	0.9 (0.6, 1.1)	76	0.9 (0.3, 1.9)		
CYP2E1 (Cytochrome P450 2E1 gene)									
rs2249695	CC	77	4 (3, 6)	15	1.0 (0.7, 1.3)	67	1.3 (0.3, 2.0)		
	CT/TT	36	5 (2.5, 6)	4	0.7 (0.4, 1.2)	30	0.8 (0.3, 1.5)		
rs8192766	TT	89	4 (3, 6)	17	0.9 (0.6, 1.1)	75	1.3 (0.4, 1.9)		
	TG/GG	24	4.5 (3, 6)	2	1.4 (1.3, 1.6)	22	0.5 (0.3, 1.3)		
rs915906	TT	85	4 (3, 6)	15	1.0 (0.7, 1.3)	74	1.3 (0.4, 2.0)		
	TC/CC	28	4.5 (2, 6.5)	4	0.7 (0.4, 1.2)	23	0.8 (0.2, 1.4)		
rs915907	CC	86	4.5 (3, 6)	16	0.9 (0.6, 1.2)	73	1.3 (0.4, 2.0)		
	CA/AA	27	4 (3, 6)	3	1.3 (0.8, 1.6)	24	0.5 (0.3, 1.4)		
rs2070673	TT	76	4 (3, 6)	15	1.0 (0.7, 1.3)	65	1.3 (0.4, 1.9)		
	TA/AA	37	5 (3, 6)	4	0.9 (0.4, 1.4)	32	0.7 (0.2, 1.4)		
rs2070676	CC	86	4 (3, 6)	16	1.0 (0.8, 1.3)	74	1.3 (0.4, 1.9)		
	CG/GG	27	5 (2, 7)	3	0.6 (0.3, 1.6)	23	0.9 (0.3, 1.5)		
rs2031920	CC	104	4 (3, 6)	18	0.9 (0.6, 1.3)	89	1.3 (0.4, 1.9) [*]		
	CT/TT	9	5 (4, 6)	1	1.3	8	0.4 (0.1, 0.6)		
rs2515641	CC	87	4 (3, 6)	16	1.0 (0.8, 1.3)	75	1.2 (0.3, 1.9)		
	CT/TT	26	5 (3, 7)	3	0.6 (0.3, 1.6)	22	1.0 (0.3, 2.3)		
GSTZ1 (glutathione S-transferase zeta 1 gene)									
rs1046428	CC	67	4 (3, 6)	11	0.9 (0.6, 1.1)	57	0.8 (0.3, 1.7) [*]		
	CT/TT	46	4.5 (3, 6)	8	1.1 (0.6, 1.5)	40	1.4 (0.8, 1.9)		
rs7972	GG	95	4 (3, 6)	17	0.9 (0.6, 1.3)	81	1.2 (0.4, 1.9)		
	GA/AA	18	4.5 (2, 5)	2	6.7 (1.0, 12.4)	16	1.1 (0.3, 1.7)		
rs7975	GG	48	4 (3, 6)	10	0.8 (0.6, 1.1)	42	1.0 (0.3, 2.0)		
	GA/AA	65	5 (3, 7)	9	1.1 (0.9, 1.3)	55	1.2 (0.4, 1.8)		
CC16 (club cell protein gene)									
rs3741240	GG							54	11.6 (9.3, 13.3) [*]
	GA/AA							54	9.5 (7.9, 13.4)

MN-PBL: Micronuclei in peripheral blood lymphocytes. MN-Ret: Micronuclei in reticulocytes.

^a At least once per month.

^b Days per week doing vigorous physical activity during leisure time (low: 0, medium: 1–2, high: > 2).

* p-Value < 0.05, calculated using Spearman correlation for continuous variables, Kolmogorov-Smirnov for dichotomous variables and Kruskal-Wallis for categorical variables with three categories.

Appendix Table A3

Effect modification by single nucleotide polymorphisms of the association between the change in micronuclei frequency in peripheral blood lymphocytes (MN-PBL) for an interquartile range increase in total (TTHM) and brominated (Br-TTHM) trihalomethanes in exhaled breath after swimming. Beta coefficients from linear regression.

Gene/polymorphism	Variant	N	TTHM	Br-TTHM
			β Coef. (95%CI)	β Coef. (95%CI)
		113	−0.47 (−1.02, 0.08)	−0.14 (−0.77, 0.49)
GSTT1 (glutathione S-transferase theta 1)	Null	23	−0.66 (−1.75, 0.42)	−0.38 (−1.62, 0.86)
	Positive	90	−0.44 (−1.08, 0.20)	−0.10 (−0.83, 0.63)
	Interaction p-value		0.791	0.765
CYP2E1 (cytochrome P450 2E1)	CC	77	−0.95 (−1.58, −0.33)	−0.87 (−1.61, −0.13)
	CT/TT	36	0.71 (−0.39, 1.82)	1.28 (0.19, 2.37)

(continued on next page)

Appendix Table A3 (continued)

Gene/polymorphism	Variant	N	TTHM	Br-TTHM
			β Coef. (95%CI)	β Coef. (95%CI)
	Interaction p-value		0.007	0.001*
rs8192766	TT	89	-0.52 (-1.12, 0.07)	-0.24 (-0.93, 0.45)
	TG/GG	24	-0.34 (-2.16, 1.47)	0.30 (-1.58, 2.17)
	Interaction p-value		0.837	0.560
rs915906	TT	85	-0.94 (-1.58, -0.29)	-0.69 (-1.46, 0.07)
	TC/CC	28	0.67 (-0.36, 1.70)	0.92 (-0.13, 1.97)
	Interaction p-value		0.010	0.018
rs915907	CC	86	-0.81 (-1.42, -0.19)	-0.59 (-1.30, 0.11)
	CA/AA	27	0.69 (-0.58, 1.95)	1.36 (0.04, 2.67)
	Interaction p-value		0.026	0.009
rs2070673	TT	76	-0.69 (-1.31, -0.06)	-0.55 (-1.32, 0.22)
	TA/AA	37	-0.01 (-1.28, 1.27)	0.53 (-0.64, 1.69)
	Interaction p-value		0.314	0.116
rs2070676	CC	86	-0.72 (-1.32, -0.13)	-0.60 (-1.32, 0.13)
	CG/GG	27	0.86 (-0.67, 2.39)	1.20 (-0.08, 2.48)
	Interaction p-value		0.043	0.014
rs2031920	CC	104	-0.41 (-0.97, 0.16)	-0.10 (-0.74, 0.55)
	CT/TT	9	-2.31 (-5.50, 0.88)	-1.01 (-4.45, 2.43)
	Interaction p-value		0.181	0.503
rs2515641	CC	87	-0.80 (-1.40, -0.20)	-0.70 (-1.42, 0.03)
	CT/TT	26	1.32 (-0.12, 2.77)	1.59 (0.41, 2.77)
	Interaction p-value		0.008	0.002*
<i>GSTZ1</i> (glutathione S-transferase zeta 1)				
rs1046428	CC	67	-0.34 (-1.05, 0.36)	-0.002 (-0.79, 0.79)
	CT/TT	46	-0.87 (-1.94, 0.20)	-0.49 (-1.70, 0.72)
	Interaction p-value		0.443	0.529
rs7972	GG	95	-0.46 (-1.04, 0.11)	-0.19 (-0.86, 0.49)
	GA/AA	18	-0.52 (-2.63, 1.59)	0.11 (-1.83, 2.05)
	Interaction p-value		0.952	0.729
rs7975	GG	48	-0.28 (-1.14, 0.58)	-0.004 (-1.00, 0.99)
	GA/AA	65	-0.55 (-1.31, 0.20)	-0.18 (-1.02, 0.66)
	Interaction p-value		0.643	0.795

* Significant p-value after Bonferroni correction, p-value at 0.004.

References

- Abramsson-Zetterberg, L., Zetterberg, G., Bergqvist, M., Grawé, J., 2000. Human cytogenetic biomonitoring using flow-cytometric analysis of micronuclei in transferrin-positive immature peripheral blood reticulocytes. *Environ. Mol. Mutagen.* 36, 22–31.
- Ainsworth, B.E., Haskell, W.L., Herrmann, S.D., Meckes, N., Bassett Jr., D.R., Tudor-Locke, C., et al., 2011. Compendium of physical activities: a second update of codes and MET values. *Med. Sci. Sports Exerc.* 43, 1575–1581.
- ASPB, Agència de Salut Pública de Barcelona, 2012. Quality of Drinking Water in Barcelona. Barcelona. Available at: http://www.aspb.cat/quefem/docs/Aigua_consum_huma.pdf.
- Bonassi, S., El-Zein, R., Bolognesi, C., Fenech, M., 2011. Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. *Mutagenesis* 26, 93–100.
- Borg, G., 1970. Perceived exertion as an indicator of somatic stress. *Scand. J. Rehabil. Med.* 2, 92–98.
- Broekaert, F., Clippe, A., Knoop, B., Hermans, C., Bernard, A., 2000. Clara cell secretory protein (CC16): features as a peripheral lung biomarker. *Ann. N. Y. Acad. Sci.* 923, 68–77.
- Cantor, K.P., Villanueva, C.M., Silverman, D.T., Figueroa, J.D., Real, F.X., Garcia-Closas, M., et al., 2010. Polymorphisms in *GSTT1*, *GSTZ1*, and *CYP2E1*, disinfection by-products, and risk of bladder cancer in Spain. *Environ. Health Perspect.* 118, 1545–1550.
- Carbonnelle, S., Francaux, M., Doyle, I., Dumont, X., de Burbure, C., Morel, G., et al., 2002. Changes in serum pneumoproteins caused by short-term exposures to nitrogen trichloride in indoor chlorinated swimming pools. *Biomarkers* 7, 464–478.
- Carbonnelle, S., Bernard, A., Doyle, I.R., Grutters, J., Francaux, M., 2008. Fractional exhaled NO and serum pneumoproteins after swimming in a chlorinated pool. *Med. Sci. Sports Exerc.* 40, 1472–1476.
- Cardador, M.J., Gallego, M., 2011. Haloacetic acids in swimming pools: swimmer and worker exposure. *Environ. Sci. Technol.* 45, 5783–5790.
- Chowdhury, S., Alhooshani, K., Karanfil, T., 2014. Disinfection byproducts in swimming pool: occurrences, implications and future needs. *Water Res.* 53, 68–109.
- Costa, C., Silva, S., Neves, J., Coelho, P., Costa, S., Laffon, B., et al., 2011. Micronucleus frequencies in lymphocytes and reticulocytes in a pesticide-exposed population in Portugal. *J. Toxicol. Environ. Health A* 74, 960–970.
- Erdinger, L., Kühn, K.P., Kirsch, F., Feldhues, R., Fröbel, T., Nohynek, B., et al., 2004. Pathways of trihalomethane uptake in swimming pools. *Int. J. Hyg. Environ. Health* 207, 571–575.
- Espín-Pérez, A., Font-Ribera, L., van Veldhoven, K., Krauskopf, J., Portengen, L., Chadeau-Hyam, M., et al., 2017. Blood transcriptional and microRNA responses to short-term exposure to disinfection by-products in a swimming pool. *Environ. Int.* 118, 42–50.
- Fenech, M., 2007. Cytokinesis-block micronucleus cytome assay. *Nat. Protoc.* 2, 1084–1104.
- Fernández-Luna, Á., Gallardo, L., Plaza-Carmona, M., García-Unanue, J., Sánchez-Sánchez, J., Felipe, J.L., et al., 2012. Respiratory function and changes in lung epithelium biomarkers after a short-training intervention in chlorinated vs. ozone indoor pools. *PLoS One* 8, e68447.
- Font-Ribera, L., Kogevinas, M., Zock, J.P., Gómez, F.P., Barreiro, E., Nieuwenhuijsen, M.J., et al., 2010. Short-term changes in respiratory biomarkers after swimming in a chlorinated pool. *Environ. Health Perspect.* 118, 1538–1544.
- Font-Ribera, L., Villanueva, C.M., Schmalz, C., Zwiener, C., Marco, E., Grimalt, J.O., et al., 2016. Environmental and personal determinants of the uptake of disinfection by-products during swimming. *Environ. Res.* 149, 206–215.
- Gängler, S., Charisiadis, P., Seth, R., Chatterjee, S., Makris, K.C., 2018. Time of the day dictates the variability of biomarkers of exposure to disinfection byproducts. *Environ. Int.* 112, 33–40.
- Gulati, M., Shaw, L.J., Thisted, R.A., Black, H.R., Merz, C.N.B., Arnsdorf, M.F., 2010. Heart rate response to exercise stress testing in asymptomatic women: the St. James women take heart project. *Circulation* 122, 130–137.
- Hery, M., Hecht, G., Gerber, J.M., Gendre, J., Hubert, G., Rebuffaud, J., 1995. Exposure to chloramines in the atmosphere of indoor swimming pools. *Ann. Occup. Hyg.* 39, 427–439.
- Holland, N., Davé, V., Venkat, S., Wong, H., Donde, A., Balmes, J.R., et al., 2015. Ozone inhalation leads to a dose-dependent increase of cytogenetic damage in human lymphocytes. *Environ. Mol. Mutagen.* 56, 378–387.
- International HapMap Consortium, 2003. The International HapMap project. *Nature* 426, 789–796.
- Kissling, G.E., Dertinger, S.D., Hayashi, M., MacGregor, J.T., 2007. Sensitivity of the erythrocyte micronucleus assay: dependence on number of cells scored and inter-animal variability. *Mutat. Res.* 634, 235–240.
- Kogevinas, M., Villanueva, C.M., Font-Ribera, L., Liviak, D., Bustamante, M., Espinoza, F., et al., 2010. Genotoxic effects in swimmers exposed to disinfection by-products in indoor swimming pools. *Environ. Health Perspect.* 118, 1531–1537.
- Llana-Belloch, S., Priego Quesada, J.I., Pérez-Soriano, P., Lucas-Cuevas, Á.G., Salvador-Pascual, A., Olaso-González, G., et al., 2015. Disinfection by-products effect on swimmers oxidative stress and respiratory damage. *Eur. J. Sport Sci.* 12, 1–9.
- Marco, E., Lourencetti, C., Grimalt, J.O., Gari, M., Fernández, P., Font-Ribera, L., et al.,

2015. Influence of physical activity in the intake of trihalomethanes in indoor swimming pools. *Environ. Res.* 140, 292–299.
- Nordberg, G.F., Lundstrom, N.G., Forsberg, B., Hagenbjork-Gustafsson, A., Lagerkvist, B.J., Nilsson, J., et al., 2012. Lung function in volunteers before and after exposure to trichloramine in indoor pool environments and asthma in a cohort of pool workers. *BMJ Open* 2, e000973.
- Plewa MJ, Wagner ED, Muellner MG, Hsu KM, Richardson SD. 2008. Comparative mammalian cell toxicity of N-DBPs and C-DBPs, in: T. Karanfil, S.W. Krasner, P. Westerhoff, Y. Xie (Eds.), *Occurrence, Formation, Health Effects and Control of Disinfection By-products in Drinking Water*. American Chemical Society, Washington, DC, Symposium Series No. 995, 36.
- Provost, E.B., Chaumont, A., Kicinski, M., Cox, B., Fierens, F., Bernard, A., Nawrot, T.S., 2014. Serum levels of club cell secretory protein (Clara) and short- and long-term exposure to particulate air pollution in adolescents. *Environ. Int.* 68, 66–70.
- Richardson, S.D., Plewa, M.J., Wagner, E.D., Schoeny, R., DeMarini, D.M., 2007. Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: a review and roadmap for research. *Mutat. Res.* 636, 178–242.
- Richardson, S.D., DeMarini, D.M., Kogevinas, M., Fernandez, P., Marco, E., Lourencetti, C., et al., 2010. What's in the pool? A comprehensive identification of disinfection by-products and assessment of mutagenicity of chlorinated and brominated swimming pool water. *Environ. Health Perspect.* 118, 1523–1530.
- Stayner, L.T., Pedersen, M., Patelarou, E., Decordier, I., Vande Loock, K., Chatzi, L., et al., 2014. Exposure to brominated trihalomethanes in water during pregnancy and micronuclei frequency in maternal and cord blood lymphocytes. *Environ. Health Perspect.* 122, 100–106.
- Stockfelt, L., Sallsten, G., Olin, A.C., Almerud, P., Samuelsson, L., Johannesson, S., et al., 2012. Effects on airways of short-term exposure to two kinds of wood smoke in a chamber study of healthy humans. *Inhal. Toxicol.* 24, 47–59.
- Stopper, H., Hempel, K., Reiners, C., Vershenya, S., Lorenz, R., Vukicevic, V., et al., 2005. Pilot study for comparison of reticulocyte-micronuclei with lymphocyte-micronuclei in human biomonitoring. *Toxicol. Lett.* 156, 351–360.
- Thomas, P., Holland, N., Bolognesi, C., Kirsch-Volders, M., Bonassi, S., Zeiger, E., et al., 2009. Buccal micronucleus cytome assay. *Nat. Protoc.* 4, 825–837.
- van Veldhoven, K., Keski-Rahkonen, P., Barupal, D.K., Villanueva, C.M., Font-Ribera, L., Scalbert, A., et al., 2018. Effects of exposure to water disinfection by-products in a swimming pool: a metabolome-wide association study. *Environ. Int.* 111, 60–70.
- Villanueva, C.M., Font-Ribera, L., 2012. Health impact of disinfection by-products in swimming pools. *Ann. Ist. Super. Sanita* 48, 387–396.
- Villanueva, C.M., Cantor, K.P., Cordier, S., Jaakkola, J.J., King, W.D., Lynch, C.F., et al., 2004. Disinfection byproducts and bladder cancer: a pooled analysis. *Epidemiology* 15, 357–367.
- Villanueva, C.M., Cantor, K.P., Grimalt, J.O., Malats, N., Silverman, D., Tardon, A., et al., 2007. Bladder cancer and exposure to water disinfection by-products through ingestion, bathing, showering, and swimming in pools. *Am. J. Epidemiol.* 165, 148–156.
- Vlaanderen, J., van Veldhoven, K., Font-Ribera, L., Villanueva, C.M., Chadeau-Hyam, M., Portengen, L., et al., 2017. Acute changes in serum immune markers due to swimming in a chlorinated pool. *Environ. Int.* 105, 1–11.