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Genotoxic Effects in Swimmers Exposed to Disinfection By-products in Indoor Swimming Pools

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Key Words: swimming pools, water, chlorination, disinfection by-products, cancer, genotoxicity, mutagenicity, genetics

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List of Abbreviations and Definitions

$CHBr_3$	Bromoform
$CHCl_2Br$	Bromodichloromethane
$CHCl_3$	Chloroform
$CHClBr_2$	Chlorodibromomethane
DBPs	Disinfection by-products
GSTT1	Glutathion-transferase theta-1
MN	Micronucleus
OTM	Olive tail moment
PBL	Peripheral blood lymphocytes
SCGE	Single Cell Gel Electrophoresis (Comet Assay)
SD	Standard deviation
THMs	Trihalomethanes

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Abstract

BACKGROUND: Exposure to disinfection by-products (DBPs) in drinking water has been associated with cancer risk. A recent study found an increased bladder cancer risk among subjects attending swimming pools relative to those not attending.

OBJECTIVES: To evaluate whether swimming in pools is associated with biomarkers of genotoxicity.

METHODS: We collected blood, urine, and exhaled air samples from 49 non-smoking adult volunteers before and after they swam for 40 min in an indoor chlorinated pool. We estimated associations between the concentrations of four trihalomethanes in exhaled breath and changes in the following biomarkers: micronuclei and DNA damage (comet assay) in peripheral blood lymphocytes before and 1 h after swimming, urine mutagenicity (Ames assay) before and 2 h after swimming, and micronuclei in exfoliated urothelial cells before and 2 weeks after swimming. We also estimated associations and interactions with polymorphisms in genes related to DNA repair or DBP metabolism.

RESULTS: After swimming, the total concentration of the four trihalomethanes in exhaled breath was seven times higher than before swimming. The change in the frequency of micronucleated lymphocytes after swimming increased in association with exhaled concentrations of the brominated trihalomethanes ($p = 0.03$ for $CHCl_2Br$, $p = 0.05$ for $CHClBr_2$, $p = 0.01$ for $CHBr_3$) but not chloroform. Swimming was not associated with DNA damage detectable by the comet assay. Urine mutagenicity increased significantly after swimming in association with the concentration of exhaled $CHBr_3$ ($p = 0.004$). No significant associations with changes in micronucleated urothelial cells were observed.

CONCLUSIONS: Our findings support potential genotoxic effects of exposure to DBPs from swimming pools. The positive health effects gained by swimming could be increased by reducing the potential health risks of pool water.

Introduction

Swimming in pools is an important recreational activity followed by hundreds of millions of people worldwide that has been associated with significant positive health benefits (Zwiener et al. 2007). Hygiene and water quality, especially infections caused by feces-associated microbes and protozoa, have been a priority for regulators and researchers (WHO 2006). However, concerns have been raised regarding potential adverse health effects resulting from exposure to chemically disinfected swimming pool water (Zwiener et al. 2007).

As with drinking water, chlorination is the most common method of disinfection for swimming pools. The addition of chlorine to water results in the formation of hundreds of chlorination by-products due to the presence of organic matter (Richardson et al. 2007). Levels of disinfection by-products (DBPs) in swimming pool water are not necessarily higher than those in drinking water (Richardson et al. 2010). Swimming in an indoor pool, however, leads to a high uptake of compounds such as trihalomethanes (THMs), which are inhaled and absorbed by the skin (Whitaker et al. 2003; Xu and Weisel 2004; Xu and Weisel 2005). High levels of haloacetic acids also have been reported in swimming pools; however, these DBPs are likely not taken up at significant levels because they are non-volatile, and uptake occurs mainly through ingestion. Another chemical class identified recently in chlorinated pools is nitrosamines (Walse and Mitch 2008), but their uptake via swimming has not been studied.

Epidemiological studies have shown that long-term consumption of chlorinated water and exposure to THMs at levels found currently in drinking water in many industrialized countries are associated with an increased risk of bladder cancer (Villanueva et al. 2004). A large study on bladder cancer in Spain was the first to examine exposure to THMs through ingestion of water and through inhalation and

dermal absorption during showering, bathing, and swimming in pools (Villanueva et al. 2007). Participants with household THM levels >49 μg/L had twice the risk of bladder cancer as those with levels <8 μg/L. The risks associated with tasks resulting in high exposure via inhalation and dermal absorption were higher than those for ingestion. In the same study, an increased risk was found for subjects attending swimming pools (odds ratio of 1.6, 95% CI 1.2 to 2.1). It has been hypothesized that increased risk in melanoma among swimmers could be partly due to disinfection by-products (Nelemans et al. 1994).

Several DBPs, including some THMs, are genotoxic and as reviewed by Richardson (Richardson et al. 2007), all four regulated THMs (chloroform, bromoform, bromodichloromethane, and chlorodibromomethane) are carcinogenic in rodents. Chloroform is not mutagenic; however, the brominated THMs are, and they are activated to mutagens by *GSTT1* (DeMarini et al. 1997; Pegram et al. 1997). Bromodichloromethane has been shown to induce mutagenic urine in humans (Leavens et al. 2007). All regulated THMs other than chlorodibromomethane have been shown to induce DNA damage in vitro detected by the comet assay, and some studies have found that chlorodibromomethane induced chromosomal aberrations and sister chromatid exchanges and that bromoform induced sister chromatid exchanges and micronuclei (MN) (IARC 1999; Richardson et al. 2007). Extensive quantitative testing of the mutagenic and genotoxic potency of DBPs has shown that iodinated compounds such as dichloroiodomethane are generally more toxic than brominated DBPs, and DBPs that are both iodinated and brominated are more genotoxic than chlorinated DBPs (Richardson et al. 2007).

Metabolism of DBPs is mediated by enzymes from the GST and CYP families. An evaluation of polymorphisms in *GSTT1*, a gene involved in the metabolism of

brominated THMs, indicated significantly stronger associations between THM exposure and bladder cancer among subjects with functioning *GSTT1* (+/+ or +/- genotypes) than among subjects with deletions in both alleles (-/-) (Cantor et al. 2010). This was consistent with early studies showing that GSTT1 activated the brominated THMs, but not chloroform, to mutagens in a transgenic strain of *Salmonella* (DeMarini et al. 1997; Pegram et al. 1997). GSTZ1 catalyzes the oxygenation of dichloroacetic acid (DCA) to glyoxylic acid and plays a critical role in the tyrosine degradation pathway and in alpha-haloacid metabolism (Board et al. 2005). *CYP2E1*, *CYP1A2*, *CYP3A4,* and *CYP2A6* are involved in the metabolism of chloroform and bromodichloromethane (Allis et al. 2002; Gemma et al. 2003; Leavens et al. 2007; Zhao et al. 2002), and *CYP2D6* variants have been found to modify THM blood levels after showering (Backer et al. 2007). Metabolic activation of bromodichloromethane by *GSTT1* may result in the formation of 8-oxoguanidine DNA adducts that are repaired by base-excision repair (BER) through the expression of genes such as *OGG1*, *APEX1,* and *XRCC1*. There exists some evidence that genetic variants of *XRCC1* influence MN formation, as might variants in the *ERCC2* gene, which is part of the nucleotide-excision repair (NER) pathway (Iarmarcovai et al. 2008). On the other hand, chloroform exposure results in increased expression of *APEX1* in rat liver (Zidek et al. 2007). Polymorphsisms in these DNA repair genes also have been associated with bladder cancer, which is the cancer that has been associated most consistently with DPB exposure.

Genotoxicity evaluations are used extensively in studies of health effects of environmental exposures. These assays can be carried out using easily obtainable human cells, such as peripheral blood lymphocytes and exfoliated urothelial cells from urine, and have been used to evaluate the genotoxicity of DBPs (Liviac et al. 2009; Ranmuthugala et al. 2003; Richardson et al. 2007). Biomarkers used to detect primary

DNA damage include the comet assay, which detects single- and double-strand breaks, alkali-labile sites, and transient DNA repair breaks (Dusinska and Collins 2008). Primary DNA damage can be repaired easily; thus, it is necessary to use also biomarkers of fixed damage, which is probably more relevant for human risk assessment. In this context, the MN assay is a well-validated methodology that provides a measure of both chromosome breakage and chromosome loss, and it has been shown to be a relevant biomarker for cancer risk (Bonassi et al. 2007).

Urinary mutagenicity has been examined in occupational and environmental settings for more than three decades as a general cost-efficient assessment of systemic genotoxicity (Cerná and Pastorková 2002). Although several experimental studies have evaluated the mutagenicity of DBPs, only one study has evaluated the effect of a DBP on urine mutagenicity in humans with controlled dermal and oral exposures (Leavens et al. 2007). The authors found that urine mutagenicity levels increased after exposure to bromodichloromethane, particularly among subjects exposed percutaneously compared to orally.

To evaluate the genotoxicity of swimming pool water in swimmers, we examined the above-mentioned biomarkers of genotoxicity in an experimental study in which adults swam for 40 min in a chlorinated, indoor swimming pool. We compared the biomarker results to an internal measure of exposure, i.e., the concentrations of four THMs in exhaled breath as determined in a companion study (Font-Ribera L et al. 2010). We also evaluated the impact of genotype on the biomarker responses relative to THM exposure.

Methods

Main design. Fifty non-smoking volunteers aged 18 to 50 years were recruited through open advertisements on the Internet and at local universities, avoiding any direct personal contact, e.g., E-mailing because this is prohibited in research centers in Spain. A screening questionnaire was used to verify eligibility. Compensation was provided to subjects, who signed an informed consent form that acknowledged this compensation. A single, indoor, 25-m long chlorinated swimming pool in Barcelona, Spain, was selected for the study. The study was conducted during May, June, September, and October 2007.. The main design of the study is shown in Figure 1.

Subjects were requested not to swim for one week prior to the swimming experiment. Subjects were asked to swim for 40 min in the pool, and the timing and distance of swimming were recorded for each individual. We selected 40 min based on our estimate of the usual time that non-competitive swimmers swim. Biological samples (exhaled breath, blood, and urine) were collected both before and after swimming at specified time periods (Font-Ribera et al. 2010), and questionnaires were completed before swimming. THMs in exhaled breath were measured within a few minutes after swimming and before taking a shower. Blood samples were collected on average 1 h after swimming, urine samples were collected on average 2 h after swimming, and a second urine sample was collected 2 weeks after swimming for the micronuclei analysis in exfoliated urothelial cells. One subject failed to complete adequately the procedures for the measurement of THMs in exhaled breath and was excluded, leaving 49 subjects for the final analysis. All laboratory analyses were blind concerning pre- or post-swimming. The study protocol was approved by the ethics committee (IRB) of the research centre, and all subjects signed an informed consent.

Questionnaire. Subjects completed a self-administered questionnaire that included information on socio-demographics (e.g., age, education, occupation, basic residential and commuting information), detailed water-related habits (e.g., fluids ingestion, showers, baths, swimming pools), physical activity, medical history and drugs, and lifestyle (e.g., past smoking, second-hand smoke, use of hair dyes), as well as a food-frequency questionnaire validated in the Spanish population, and a short 24-h activity and recent-disease questionnaire. Measurement of physical activity during swimming was done indirectly by recording each individual's swimming pattern (number of laps).

Exposure assessment. A detailed description of measurements of THMs in air and exhaled breath before and after swimming is reported in a companion paper (Font-Ribera et al. 2010), along with information on levels of DBPs in the water and water mutagenicity (Richardson et al. 2010). THMs in air and water were determined at each swimming session following a specific protocol. Exhaled breath has distinct advantages for the assessment of THM intake. It is non-invasive and provides a representative estimate of the concentration of contaminants in blood due to the gas exchange in the blood/breath interface in the lungs. Air and exhaled breath involved solid-phase adsorption on Tenax TA. Exhaled breath samples were collected using a portable system that consisted of a Haldane-Priestley tube modified to concentrate aliquots of exhaled breath from one or more exhalations. Water samples were analyzed with a purge-and-trap concentrator equipped with a Tenax® silica gel-charcoal trap.

Comet analysis in peripheral blood lymphocytes. The comet assay was performed as described previously (Singh et al. 1988) with minor modifications. Blood samples were collected in vacutainers with EDTA. Samples were kept chilled, and the length of time between blood collection and sample processing was a few hours

(Anderson et al. 1997). One hundred cells selected randomly (50 cells from each of the two replicate slides) were analyzed per sample. Olive tail moment (OTM) and percentage of DNA in the tail were used as measures of DNA damage and computed using Komet Version 5.5 software (see also Supplemental Material).

MN analysis in peripheral blood lymphocytes. Blood was obtained from each subject by venipuncture using heparinized vacutainers and sent immediately to the laboratory for the lymphocyte cultures. To determine the frequency of binucleated cells with micronuclei (BNMN) and the total number of micronuclei, a total of 1000 binucleated cells with well-preserved cytoplasm (500 per replicate) were scored for each subject. In addition, 500 lymphocytes were scored to evaluate the percentage of cells with one to four nuclei, and the cytokinesis block proliferation index (CBPI) was calculated (Surrallés et al. 1995). Microscopic scoring was performed on coded slides (see also Supplemental Material).

MN analysis in urothelial cells. Urine samples were collected before swimming and again 2 weeks later in plastic vials (~50 mL) and sent to the laboratory where they were processed the same day. Two weeks was selected because this amount of time is required for exfoliation of cells from the urothelium (Espinoza et al. 2008). The criteria for MN evaluation were those suggested by Stick (Stick et al. 1983) as updated by subsequent guidelines by Fenech (Fenech et al. 2003). The frequency of urothelial cells with MN and the total number of MN were determined for each analyzed subject. Only those cells with a typical morphology corresponding to the urothelial cells were scored. This criterion avoids any kind of bias, especially in women where many squamous cells not of urothelial origin are observed. Although bacteria were present in a few urine samples, they did not interfere with the scoring (see also Supplemental Material).

Urine mutagenicity. Urine samples (30 mL) collected prior to and 90 to 120 min after exposure were evaluated for mutagenicity in the *Salmonella* (Ames) mutagenicity plate-incorporation assay (Maron and Ames 1983) in strain YG1024 with S9 mix. YG1024 is a frameshift strain derived from TA98 (*hisD3052,* Δ*uvrB, rfa,* pKM101) that contains acetyltransferase activity (Watanabe et al. 1990); it has been used extensively for urinary mutagenicity studies (Cerná and Pastorková 2002). Only one strain was used because of the limited availability of sample. The mutagenic potencies of samples, expressed as revertants (rev) per mL-equivalent, were calculated from the slope of the regression over the linear portion of the dose-response curves. Slopes were calculated for only 43 subjects with samples from before and after swimming that were sufficient for analyses of mutagenicity at ≥3 different concentrations (see also Supplemental Material).

Gene selection and genotyping. We examined genetic variants, including single-nucleotide polymorphisms (SNPs), and copy-number variants (CNVs) in three genes involved in the metabolism of DBPs (*GSTT1, CYP2E1,* and *GSTZ1),* four additional genes that may play a minor role in the metabolism of DBPs (*GSTT2B, GSTM1, CYP1A2* and *CYP2D6*), and four DNA repair genes that could be relevant when examining results, particularly for the comet assay (*APEX1, ERCC2, OGG1, XRCC1*). We selected tagSNPs combined with functional variants most likely to influence gene expression or function. In total, three CNVs and 17 SNPs were genotyped. For a complete list, see Supplemental Material, Tables 1-4.

SNP genotyping was performed using the Sequenom® platform. Two individuals with low genotyping frequency (<75%) and two non-Caucasians were excluded from the genetic analyses. Genotyping failed completely for the following SNPs: rs11101815 and rs915908 in *CYP2E1*, rs1799793 in *ERCC2,* and rs28903081 in

the *XRCC3* gene. The remaining 13 SNPs had a call frequency >90% (see also Supplemental Material).

Statistical analysis. Paired t-tests were used to examine changes in the level of biomarkers before and after swimming. Associations between exposure to THMs measured in exhaled breath after swimming and changes in markers of genotoxicity before and after swimming were evaluated using linear regression. All analyses were adjusted for age and sex. Several other variables were evaluated as potential confounders, including water consumption, source of water, antioxidant intake from diet, number of laps swum during the experiment (an indication of physical activity), and leisure-time physical activity. However, because estimated effects were modified only marginally by the inclusion of these variables in the models, we report results adjusted for age and sex only. In order to estimate the amount of variance in an endpoint due to the concentration of THMs in exhaled breath, we used unadjusted models to calculate the r^2 values. The default *p*-value used to determine statistical significance was <0.05.

We also tested for genotype deviations from Hardy-Weinberg equilibrium (HWE) (Wigginton et al. 2005). Analysis of single-marker effect was performed assuming both a dominant and an additive (not shown) genetic model, considering the most frequent allele as a reference category, and using logistic regression implemented in the SNPassoc package (version 1.5-1) from R statistical software (Version 2.6.1) (R Development Core Team 2007). To evaluate interactions between changes in THMs and gene variants, we included an interaction term for dichotomous genotype and THMs in the linear regression models.

Results

Among the 50 subjects who participated in at least part of the study, 66% were women, 96% were Caucasian, most were highly educated, and by selection criteria, all were non-smokers, with approximately one-third being ex-smokers (Table 1). A high percentage was exposed regularly to second-hand smoke. About half did regular sports (once/week), and 11 (22%) swam at least once/month. The average free chlorine level in the pool water was 1.17 mg/L (standard deviation, SD = 0.4), and the average total THM levels were 45.4 μg/L (SD = 7.3). In pool air the average total THM levels were 74.1 μg/ m^3 (SD = 23.7). One subject failed to complete adequately the procedures for the measurement of THMs in exhaled breath and was excluded, leaving 49 subjects for the final analysis. In exhaled breath, THM levels increased, on average, about 7 times during swimming. The average total THM levels before and after swimming were 1.2 and 7.9 μg/m^3 respectively, and the corresponding average levels for the individual THMs were 0.7 and 4.5 μg/m^3 for chloroform, 0.26 and 1.78 μg/m^3 for bromodichloromethane, 0.13 and 1.2 μg/m^3 for chlorodibromomethane, and 0.1 and 0.5 μg/m^3 for bromoform (tribromomethane).

The average number of MN positive cells per 1000 binucleated lymphocytes increased non-significantly from 3.4 before swimming to 4.0 after swimming (Table 2). Likewise, the average frequency of MN in urothelial cells and the level of urinary mutagenicity also increased non-significantly after swimming relative to before swimming. In contrast, we observed a small but statistically significant decrease in the average amount of DNA damage in blood lymphocytes after swimming relative to before swimming (Table 2).

In the multivariate analysis, the change in the frequency of MN in peripheral blood lymphocytes before and after swimming was associated with the combined

concentration of all four THMs measured in exhaled breath. Specifically, a 1 μg/m^3 increase in total THMs in exhaled breath after swimming was associated with an average increase of 0.296 MN per 1,000 cells; however, this increase was not significant (p = 0.09) (Table 3). The largest increases in MN were observed for the brominated THMs (Table 3), with statistically significant increases associated with exposure to bromodichloromethane [1.9 MN per 1000 cells, 95% confidence interval (CI) 0.21, 3.63] and bromoform (5.04 MN per 1000 cells, 95% CI 1.23, 8.84). Based on the r^2, the fraction of the variance in changes in MN frequency that was explained by exposure to bromodichloromethane was 10%; for bromoform this was 13%. Adjustment for potential confounders, including the number of laps swum during the experiment (as measure of physical activity), resulted in only minor changes in effect estimates compared with those adjusted for age and sex only (see Supplemental Material, Figure 1).

Total or individual THM concentrations in exhaled breath were not associated with the level of DNA damage in peripheral blood lymphocytes as assessed by the comet assay, regardless of whether damage was quantified based on the OTM (Table 3) or the percent of DNA in the tail (data not shown).

MN frequency in exfoliated urothelial cells was increased with a 1 μg/m^3 increase in $CHClBr_2$ (2.4 MN per 2000 cells, 95% CI -3.3, 8.2) and $CHBr_3$ (4.3 MN per 2000 cells, 95% CI -6.9, 15.5), but estimates were not statistically significant (Table 3).

An increase in urine mutagenicity, measured as an increase in the slope of the dose-response curve (mutagenic potency) before and approximately 1.5 h after swimming, was observed for the combined concentration of the four THMs in exhaled breath as well as for the concentration of each individual THM (Table 3). However, these increases were statistically significant only for bromoform (5.27, 95% CI 1.80,

8.75, $p = 0.004$). Based on the r^2, the fraction of the variance in change in urine mutagenicity that was explained by exposure to bromoform was 16%.

Modification of estimated effects of THMs on measured outcomes by genetic variation in specific genes involved in the metabolism of DBPs or in DNA repair was evaluated by modeling interactions between dichotomous genotypes and changes in THMs with swimming. Results of models to assess interactions between bromoform (the THM showing the most significant associations with the effect biomarkers) and polymorphisms in *GSTT1*, *GSTZ1* and *CYP2E1* metabolism genes are shown in Table 4. Complete results for all gene variants examined are reported in Supplemental Material, Tables 1, 2, 3 and 4.

Subjects with the null *GSTT1* genotype (-/-, a deletion in both copies of the gene) had lower frequencies of MN in urothelial cells and lower urinary mutagenicity than those with one (+/-) or none (+/+) of the copies being deleted, but differences were not statistically significant (Table 4). We did not observe a statistically significant modification by *GSTT1* of effects of THMs on MN in peripheral blood lymphocytes, although -/- individuals tended to have higher MN levels in lymphocytes compared to -/+ and +/+ individuals. Statistically significant interactions were found between exposure to bromoform and *GSTZ1* (rs3177427) for MN in blood (10.2, 95% CI 3.0, 17.3 and 1.38 MN per 1000 cells, 95% CI -3.7, 6.5 for GG versus AG or AA genotypes, respectively) and between bromoform and *CYP2E1* (rs915906) for MN in urine (11.6, 95% CI 2.5, 20.8 and -23.2, 95% CI -52.4, 6.1 for TT versus CT or CC genotypes, respectively) (Table 4). Statistically significant interactions between bromoform and gene variants on MN in peripheral lymphocytes were also estimated for *GSTT2B* (9.6, 95% CI -0.11, 19.3 and 1.95, 95% CI -2.32, 5.51 for +/+ versus -/+ or -/- genotypes, respectively), and *APEX1* (9.5, 95% CI 3.3, 15.6 and -2.5, 95% CI -9.4, 4.5 for TT

versus GT or GG genotypes, respectively) (Supplemental Material, Table 1) and between bromoform and *GSTM1* on MN in urine (-23.1, 95% CI -50.5, 4.5 and 8.1, 95% CI -2.1, 18.3 for -/- versus -/+ or +/+ genotypes, respectively) (Supplemental Material, Table 2). No statistically significant interactions were found between bromoform and any of the gene variants on DNA damage assessed using the comet assay (Table 4 and Supplemental Material, Table 4).

Discussion

This is the first study of the genotoxicity of exposure to DBPs among swimmers in a chlorinated pool. Biomarkers of genotoxic effects have been used extensively to evaluate potential health effects of environmental exposures, and the MN assay has been shown to be a predictive biomarker of cancer risk within a population of healthy subjects (Bonassi et al. 2007). Changes in biomarkers of genotoxicity (MN in blood and urinary mutagenicity) after swimming were increased with exposure to brominated THMs (determined based on the change in brominated THM concentration in exhaled breath after swimming), but not in association with exposure to chloroform, which is not genotoxic (Richardson et al. 2007). There was no association between exposure to THMs in the pool and DNA damage in blood lymphocytes as measured by the comet assay. Associations were not dependent on confounding factors. There was some indication that responses to THMs were modified by variation in genes that metabolize these compounds, but there was limited power to evaluate gene-environment interactions.

The four THMs we evaluated are the most common DBPs in swimming pool water (Richardson et al. 2010). Although the THMs are not considered to be the most toxic of the DBPs, all four are carcinogenic in rodents (Richardson et al. 2007). The brominated THMs have been shown to be mutagenic after activation by *GSTT1-1,* and some of them have been shown to induce chromosomal aberrations, sister chromatid exchanges, and/or MN in animal and human cells (IARC 1999; Richardson et al. 2007). In contrast, chloroform is not genotoxic (Richardson et al. 2007), and unlike the brominated THMs, it is not activated by *GSTT1-1* (Pegram et al. 1997). Thus, our finding of an association between exposure to brominated THMs and an increased

response among various genotoxicity biomarkers, but the absence of such an association with chloroform exposure, is consistent with the toxicology of these THMs.

Our results are also consistent with extensive quantitative genotoxicity data on DBPs showing that brominated DBPs are generally more genotoxic and carcinogenic than chlorinated DBPs (Plewa et al. 2008; Richardson et al. 2007). In our study, we evaluated only THMs that are known to be the most common DBPs in swimming pool water and that exhibit high uptake by swimmers (Zwiener et al. 2007). Levels of haloacetic acids also can be high in swimming pools; however, the uptake of these DBPs may be low because the haloacetic acids are not volatile and are not adsorbed efficiently by the skin (Xu et al. 2002). Among the chemical classes of DBPs, the rank order of the combined cytotoxicity and genotoxicity in CHO cells was halonitromethanes > haloacetamides > haloacetonitriles > haloacetic acids > halomethanes (Richardson et al. 2007). Future studies of swimmers should evaluate more completely the uptake and potential effects of a range of DBPs and other compounds present in pool water.

Evaluation of modification of the environmental exposure by genetic polymorphisms was of low statistical power given the relatively small sample size, and interpretation of these findings should be done with caution. The main hypotheses focused on a potential modification of the effect by variants in a few genes (*GSTT1*, *GSTZ1*, *CYP2E1*) that code enzymes that are important for DBP metabolism (Richardson et al. 2007). Similar to what has been shown for mutagenesis in bacteria and DNA adducts in rodents (DeMarini et al. 1997; Pegram et al. 1997; Ross and Pegram 2003), individuals with the *GSTT1* null genotype had lower frequencies of MN in urothelial cells and lower urinary mutagenicity than those with at least one functional allele; however, in our case, the differences were not statistically significant. We did

not observe modification by *GSTT1* of effects of THMs on MN in peripheral blood lymphocytes, consistent with a lack of *GSTT1* expression in lymphocytes (Wang et al. 2000). In contrast, individuals bearing *GSTT2B* +/+ had higher numbers of MN in lymphocytes than other subjects. The CNV encompassing *GSTT2B*, which modifies *GSTT2* gene expression, is in linkage disequilibrium with the *GSTT1* CNV (Zhao et al. 2009). All three of these genes are located in the same cluster, and combined effects cannot be excluded; however, the role of *GSTT2* and *GSTT2B* genes on DBP detoxification is unknown. Limited experimental data are available for *GSTZ1* and *CYP2E1* in relation to DBP exposure. In this study, we identified differences between subjects of different genotypes, with those associated with *CYP2E1* being statistically significant. These findings should be verified in further studies.

Potential confounding was minimized in our study, which involved a comparison of individuals to themselves before and after an exposure over a limited time period. Any changes in biomarkers were likely attributed to one of three factors: swimming pool related exposures, swimming itself or chance. Control of other lifestyle factors and environmental exposures, as expected, did not modify results. Physical activity has been shown in some studies (Schiffl et al. 1997) to be associated with genotoxicity through an effect on oxidative stress, but the results are not consistent (Battershill et al. 2008; Stephanie et al. 2008). In our study population, adjustment in the analysis for the intensity of physical activity during swimming did not confound effect estimates for exposure to DBPs.

Confounding could be more of a problem for the analysis of MN in exfoliated urothelial cells, which were collected two weeks after swimming. Although we did control for several lifestyle factors in the analysis, it is still possible that results in urine could have been affected more by uncontrolled confounding. Chance could be an

explanation for some of the results and is particularly a problem for the evaluation of gene-environment interactions. In the main analyses of exposure and effect biomarkers, however, only a few comparisons were done, and the issue of chance findings due to multiple comparisons was minor. In addition, the identification of more pronounced genotoxic effects for the potentially more toxic brominated compounds compared to chloroform argues against an effect of chance.

The timing of the collection of biological samples is crucial when evaluating biomarkers of effect. Due to constraints in the study protocol, the first collection of samples had to be done during a 2-h period after swimming and 2 weeks after swimming for exfoliated urothelial cells. Due to the lack of previous studies of this type with swimmers, we had no precedence to follow, and the timing of blood collection that we used might not have been the most appropriate for some of the assays. Specifically, it is possible that collecting blood approximately 1 h after swimming for chemicals that are metabolized rapidly and that are of relatively low toxicity might not necessarily be appropriate for the comet assay because DNA damage induced by DBPs may already have been repaired before the sample is collected (Komaki et al. 2009; Liviac et al. 2009). However, studies in rodents frequently assess DNA damage in lymphocytes 3-4 h after exposure. We collected urine 2 h after swimming for mutagenicity analysis and also two weeks after swimming for MN analysis. This last time period was selected to allow time for the exfoliation of cells from the urothelium exposed at the time of the experiment (Espinoza et al. 2008).

Swimming has significant positive health effects related to the benefits of exercise and has some advantages over land-based activities for people of all ages and physical abilities (Zwiener et al. 2007). To retain the positive aspects of aquatic activities, regulators and researchers have turned their attention to the hygienic aspects

of the quality of pool water, as well as of its chemical composition. It will be important to maintain microbial disinfection while minimizing potentially harmful DBPs. The goal would be to maintain the positive health effects of swimming through exercise while reducing other potential adverse health risks.

In conclusion, we found that exposure to brominated THMs through swimming in pools was associated with increases in genotoxicity biomarkers. Our findings were consistent when we examined different genotoxicity and mutagenicity assays, and we found that only brominated THMs were associated with higher genotoxicity; chloroform was not. The results are also consistent with the presence of mutagenic and genotoxic DBPs in pool water and the mutagenic activity of the pool water (~1200 rev/L-eq in strain TA100 of *Salmonella*), which, as noted by Richardson et al. (2010) were present at levels similar to those found in drinking water. However, the concentrations of nitrogen-containing DBPs were higher in pool water than in drinking water. Although our study had low power to estimate unambiguously the effects of genetic variation on responses to chemical exposures during swimming, it appears plausible that such variation exists. Our findings, which should be verified in larger studies, indicate that the positive health effects gained by swimming could be increased by reducing the potential heath risks of pool water.

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Table 1. Characteristics of the study population

Characteristic	Number	%
Sex		
Men	17	34
Women	33	66
Age (mean ± SD)	30.1 ± 6.1	
Age range	20 - 51	
Ethnicity		
Caucasian	48	96
Other	2	4
Education[a]		
Secondary	4	8
University	45	92
Tobacco		
Never smokers	36	72
Ex-smokers	14	28
Second-hand smoke		
Yes	34	68
No	16	32
Regular swimming (≥ once/month)		
Yes	11	22
No	39	78
Regular sport (≥ once/week)		
Yes	27	54
No	23	46

[a]Missing value for one subject.

Table 2. Change in mean values of biomarkers before and after swimming, peripheral blood lymphocytes (PBL) and urine.

	No. of subjects		Mean value ± SD		
Biomarker[a]	Before	After	Before	After	*p*-value[b]
MN-PBL	49	49	3.4 ± 2.4	4.0 ± 2.8	0.235
OTM-Comet-PBL	49	49	1.5 ± 0.7	1.3 ± 0.6	0.008
MN-urothelial cells	33	33	9.0 ± 9.3	10.3 ± 7.4	0.350
Urine-mutagenicity	43	43	0.6 ± 2.3	1.2 ± 2.2	0.257

[a]MN-PBL: Micronucleated cells per 1000 binucleated cells; OTM-Comet-PBL: Olive tail moment per 100 cells; MN-urothelial cells: micronucleated cells per 2000 cells; Urinary mutagenicity: Rev/ml-eq.

[b]Paired t-test.

Table 3. Changes in micronuclei (MN) frequency and olive tail moment (OTM) in peripheral blood lymphocytes (PBL), MN frequency in exfoliated urothelial cells and urine mutagenicity with a 1 μg/m^3 increase in total or individual trihalomethanes (THMs) in exhaled breath

Biomarker[a]	Exposure	Beta-coefficient[b]	95 % confidence interval	*p*-value
MN-PBL	Total THMs	0.30	-0.05 - 0.64	0.09
	$CHCl_3$	0.29	-0.27 - 0.85	0.31
	$CHCl_2Br$	1.92	0.21 - 3.63	0.03
	$CHClBr_2$	1.71	-0.02 - 3.44	0.05
	$CHBr_3$	5.04	1.23 - 8.84	0.01
OTM-Comet-PBL	Total THMs	-0.02	-0.07 - 0.04	0.53
	$CHCl_3$	-0.02	-0.10 - 0.06	0.64
	$CHCl_2Br$	-0.04	-0.30 - 0.23	0.79
	$CHClBr_2$	-0.14	-0.40 - 0.13	0.30
	$CHBr_3$	-0.23	-0.83 - 0.37	0.45
MN-urothelial cells	Total THMs	-0.10	-1.19 - 1.01	0.86
	$CHCl_3$	-0.47	-2.11 - 1.18	0.57
	$CHCl_2Br$	-0.46	-6.50 - 5.58	0.88
	$CHClBr_2$	2.44	-3.32 - 8.20	0.40
	$CHBr_3$	4.29	-6.87 - 15.45	0.44
Urine mutagenicity	Total THMs	0.24	-0.11 - 0.58	0.17
	$CHCl_3$	0.33	-0.22 - 0.89	0.23
	$CHCl_2Br$	0.61	-1.12 - 2.35	0.48
	$CHClBr_2$	0.92	-0.75 - 2.59	0.27
	$CHBr_3$	5.27	1.80 - 8.75	0.004

[a]MN-PBL: Micronucleated cells per 1000 binucleated cells; OTM-Comet-PBL: Olive tail moment per 100 cells; MN-urothelial cells: micronucleated cells per 2000 cells; Urinary mutagenicity: Rev/ml-eq.
[b]Beta-coeffficients represent a change in the biomarker level for a 1 μg/m^3 change in THMs in exhaled air measured after swimming.

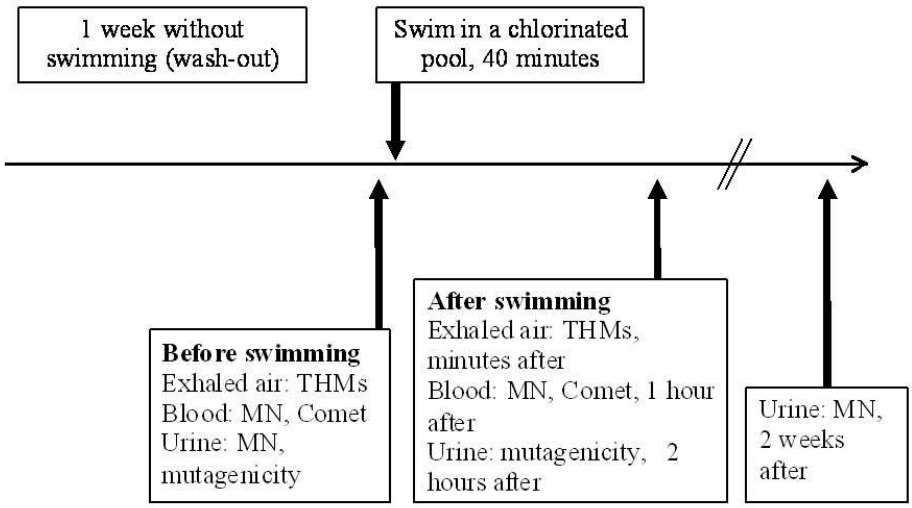
Table 4. Interaction between the effect of exposure to bromoform and polymorphisms in three genes involved in DPB metabolism on micronuclei and the comet assay in peripheral blood lymphocytes, micronuclei in exfoliated urothelial cells, and urine mutagenicity *

		Micronuclei in lymphocytes				Micronuclei in exfoliated urothelial cells				Urine mutagenicity				Comet assay			
Gene	Geno-types	n	Beta-coefficient	95%CI		n	Beta-coefficient	95%CI		n	Beta-coefficient	95%CI		n	Beta-coefficient	95%CI	
GSTT1	-/-	16	7.8	1.9	13.7	10	-5.2	-48.6	38.3	14	1.9	-4.2	8.0	16	-0.04	-0.8	0.7
	-/+, +/+	30	3.4	-1.6	8.3	23	1.7	-11.1	14.6	26	7.6	3.0	12.2	30	-0.4	-1.3	0.4
CYP2E1																	
rs2070673	TT	27	4.4	-0.4	9.1	21	10.6	0.9	20.2	22	6.9	0.7	13.0	27	-0.3	-1.1	0.5
	AT_AA	16	-0.03	-7.5	7.5	10	-11.5	-41.1	18.1	15	3.0	-2.9	9.0	16	0.05	-1.3	1.4
rs915906	TT	28	6.7	2.2	11.1	22	**11.6**	**2.5**	**20.8**	22	5.8	1.8	9.9	28	-0.4	-1.03	0.2
	CT/CC	14	-5.9	-14.1	2.3	9	**-23.2**	**-52.4**	**6.1**	14	3.1	-4.0	10.2	14	-0.1	-2.2	2.0
rs915907	CC	30	3.7	-1.8	9.1	22	12.1	-1.3	25.5	27	4.9	-0.9	10.6	30	0.4	-0.5	1.3
	CA_AA	14	6.6	-2.0	15.1	9	9.8	-12.3	31.9	11	5.4	-5.1	15.8	14	0.9	-1.7	-0.04
rs2515641	CC	35	5.8	1.7	9.9	25	7.6	-3.2	18.4	29	5.5	1.1	10.0	35	-0.3	-0.9	0.3
	CT	9	-0.4	-14.1	13.3	6	-11.0	-23.6	1.6	9	2.9	-9.7	15.5	9	0.8	-2.0	3.5
rs2249695	CC	25	4.2	-0.8	9.2	21	10.6	0.9	20.2	21	7.3	2.8	11.9	25	-0.3	-1.1	0.5
	CT_TT	19	7.5	1.1	13.8	10	-11.5	-41.1	18.1	17	2.5	-2.7	7.6	19	-0.1	-1.1	0.8
GSTZ1																	
rs3177427	GG	22	**10.2**	**3.0**	**17.3**	19	-0.2	-19.4	19.0	21	3.5	-1.8	8.7	22	-0.2	-1.2	0.7
	AG_AA	22	**1.4**	**-3.7**	**6.5**	12	8.7	-5.6	22.9	17	8.7	2.0	15.4	22	-0.5	-1.5	0.5
rs1046428	CC	29	3.3	-1.0	7.5	20	5.8	-5.7	17.2	24	6.5	1.9	11.2	29	-0.3	-1.1	0.4
	CT_TT	14	-5.4	-18.5	7.8	11	21.1	-7.6	49.8	13	-2.1	-18.9	14.7	14	0.6	-1.9	3.2

[a]Statistically significant interactions ($p < 0.05$) in **bold.**

Figure legend

Figure 1. Main design of the swimming pool study. Samples were collected before and after swimming in a period of minutes (exhaled breath), 1 h (blood for micronuclei and comet), 2 h (urine for mutagenicity testing), and 2 weeks (urine for micronuclei in exfoliated cells).


1 week without swimming (wash-out)
Swim in a chlorinated pool, 40 minutes
Before swimming
Exhaled air: THMs
Blood: MN, Comet
Urine: MN, mutagenicity
After swimming
Exhaled air: THMs, minutes after
Blood: MN, Comet, 1 hour after
Urine: mutagenicity, 2 hours after
Urine: MN, 2 weeks after