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Gene expression changes in blood RNA after swimming in a chlorinated pool

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ABSTRACT

Exposure to disinfection by-products (DBP) such as trihalomethanes (THM) in swimming pools has been linked to adverse health effects in humans, but their biological mechanisms are unclear. We evaluated short-term changes in blood gene expression of adult recreational swimmers after swimming in a chlorinated pool. Volunteers swam 40 min in an indoor chlorinated pool. Blood samples were drawn and four THM (chloroform, bromodichloromethane, dibromochloromethane and bromoform) were measured in exhaled breath before and after swimming. Intensity of physical activity was measured as metabolic equivalents (METs). Gene expression in whole blood mRNA was evaluated using IlluminaHumanHT-12v3 Expression-BeadChip. Linear mixed models were used to evaluate the relationship between gene expression changes and THM exposure. Thirty-seven before-after pairs were analyzed. The median increase from baseline to after swimming were: 0.7 to 2.3 for MET, and 1.4 to 7.1 μg/m^3 for exhaled total THM (sum of the four THM). Exhaled THM increased on average 0.94 μg/m^3 per 1 MET. While 1643 probes were differentially expressed post-exposure. Of them, 189 were also associated with exhaled levels of individual/total THM or MET after False Discovery Rate. The observed associations with the exhaled THM were low to moderate (Log-fold change range: –0.17 to 0.15). In conclusion, we identified short-term gene expression changes associated with swimming in a pool that were minor in magnitude and their biological meaning was unspecific. The high collinearity between exhaled THM levels and intensity of physical activity precluded mutually adjusted models with both covariates. These exploratory results should be validated in future studies.

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Introduction

Disinfection by-products (DBPs) are undesired toxicants produced by the reaction of organic matter with disinfectants used during water treatment, which could potentially generate deleterious health effects. Swimming in chlorinated pools involves a high exposure to DBPs through inhalation and dermal absorption (Hlavsa et al., 2011; Kim et al., 2002). Acute and subacute exposures to airway irritants in swimming pools have been associated to upper respiratory symptoms (Bernard et al., 2009). Use of chlorinated swimming pools is associated with an increase in upper tract respiratory symptoms and asthma prevalence among recreational and elite swimmers but not in children (Fernández-Luna et al., 2015; Ferrari et al., 2011; Goodman and Hays, 2008; Villanueva and Font-Ribera, 2012; Weisel et al., 2009). Chronic exposure to DBPs in drinking water has been consistently related to bladder cancer (Costet et al., 2011). In a Spanish case control study, swimming pool attendance was also linked to an increased bladder cancer risk (Villanueva et al., 2007).

In swimming pools, DBPs are formed by chlorination of several human organic materials (hair, lotion, saliva, skin and urine) (Kim et al., 2002). Among DBP classes, trihalomethanes (THM) are one of the most common in chlorinated drinking water and swimming pools (Catto et al., 2012; Richardson et al., 2010). Although total THM levels or the individual compounds (*e.g.* chloroform, bromodichloromethane, dibromochloromethane, and bromoform) may not reflect the DBP mixture composition, they have been used as a proxy of DBPs exposure in epidemiological studies (Villanueva et al., 2015; Villanueva and Font-Ribera, 2012). Levels of THM in exhaled air has been used as a short-term internal dose biomarker reflecting the alveolar/blood levels of these volatile compounds in the lung interface (Lourencetti et al., 2010; Pleil and Lindstrom, 1997). There are no available valid biomarkers to irritant volatile DBPs such as chloramines and bromamines due to its short half-life on the airway and potential confounding for *in vivo* production by neutrophils and eosinophils during inflammation (Li and Blatchley, 2007; Senthilmohan et al., 2008; Storer et al., 2011).

Biological mechanisms underlying the association between DBP exposure and chronic and acute adverse health effects are unclear. Several biological mechanisms have been proposed for chronic exposure to THM from animal studies, such as genotoxicity or oxidative stress (Nieuwenhuijsen et al., 2009; Villanueva et al., 2015). However, given that most of these toxics have a short half-life (hours to days) and only a few bioaccumulate during short periods, repeated cycles of cell cytotoxicity and regeneration are a plausible alternative to explain their toxicity (Golden et al., 1997). Data derived from human small intestine epithelial cells have shown gene expression alterations after acute exposures (30 min) to monohaloacetic acids (Attene-Ramos et al., 2010).

Swimming pools are the closest to a natural short-term DBP exposure experiment in humans. In 2007, an experimental study explored biological mechanisms of the DBP exposure in swimming pools using several biomarkers of respiratory and genotoxicity effects (Font-Ribera et al., 2010; Kogevinas et al., 2010). In this study, the bromoform uptake after swimming, was related to an increase of serum CC16 (a biomarker of lung epithelium damage) (Font-Ribera et al., 2010), micronuclei frequency (in blood lymphocytes), and urine mutagenicity (Font-Ribera et al., 2010; Kogevinas et al., 2010). Simultaneously, exercise intensity during swimming was related to an increase of both serum CC16 levels and to the uptake of THM. As THM are volatile non-polar compounds, inhalation and dermal absorption are the main incorporation routes in swimming pools (Erdinger et al., 2004), and intensity of physical activity is a potential confounder, as increased breathing rates, breathing depth, and skin vasodilatation may increase the THM uptake (Aggazzotti et al., 1998; Lourencetti et al., 2012; Marco et al., 2015; Nieuwenhujsen and Droz, 2004).

We aim to provide short-term mechanistic information studying the changes in whole-genome expression patterns in adult recreational swimmers before and after 40 minute swimming in a pool attributable to the gain in the THM levels in exhaled and change in their metabolic rate levels. These are new data from the previous study using targeted respiratory and genotoxicity biomarkers (Font-Ribera et al., 2010; Kogevinas et al., 2010).

1. Experimental

1.1. Study design and population

We conducted an experimental before–after exploratory design in nonsmoking adult volunteers in Barcelona, Spain. Volunteers were recruited through open advertisements at local universities and were screened to verify eligibility (adult non-smokers, without any respiratory/allergic symptoms in the previous 3 weeks – including cold symptoms – they should have never been diagnosed as asthmatic). Per protocol, all the participants should abstain from swimming in pools during the week before and from taking a shower on the day of the experiment. The volunteers should swim at their own pace during 40 min in a single indoor 25-m-long chlorinated swimming pool. Participants were evaluated between 09:00 and 14:00 hr (before lunch) during 19 days in May, June, September, or October 2007. Biological samples and other measurements were obtained in a room at the sports center outside from the swimming pool area.

In total, 34 volunteers were recruited for the study. To increase variability in exposure, 8 volunteers were asked to remain in the water without physical activity. A subset of 4 subjects was evaluated twice to discard potential carry-over effects of the biomarker of exposure and as a cross-over validation under a fixed non-exercise conditions (further details are available Appendix A methods S1, and Table S2). Among the 34 recruited subjects, one sample was mislabeled and the subjects finally included in the analyses were 33. These led to 74 samples analyzed (37 before–after pairs). The study was approved by the Ethics Committee of the Research Center according to National and International Regulations. The volunteers signed an informed consent form before participation.

1.2. Personal data and biological samples

Weight and height were measured with standard procedures. Body mass index was calculated as the weight in kilograms over the squared height in meters. Blood samples were collected before and 1 hr after swimming according to a strict schedule previously described (Font-Ribera et al., 2010) in PAXgene tubes (PreAnalytiX, Switzerland) and stored at −80°C. The total number of available RNA samples was 38 before–after pairs.

1.3. Biomarkers of exposure and environmental measurements

Four trihalomethanes in exhaled breath were measured as proxies of short-term individual exposure to DBPs, before and immediately after swimming using a three-way valve sampling device (Lourencetti et al., 2010). Briefly, volunteers were required to breathe twice into the device mouth-piece to purge the air present in the system. A volume of 1 L was collected per person. Chloroform, bromodichloromethane, dibromochloromethane, and bromoform were determined by an Automatic Thermal Desorption System (ATD 400; Perkin-Elmer, Shelton, CT, USA) coupled to an Autosystem gas chromatograph with electron capture detection (Perkin-Elmer). Concentrations were expressed in micrograms per cubic meter ($\mu g/m^3$).

The four trihalomethanes were also measured in the air and water of the swimming pool following procedures previously described (Lourencetti et al., 2010, 2012). In brief, water samples were collected in 40 mL amber glass vials headspace-free with 3 mg of sodium thiosulfate as quencher and analyzed by gas chromatography coupled to a mass spectrometer. Results of these characterizations have been published previously (Richardson et al., 2010).

1.4. Metabolic rate

Intensity of physical activity was measured as the corrected metabolic equivalents (MET) (Ainsworth et al., 2011; Shono et al., 2001). The resting metabolic rate and the adjusted metabolic rate per subject were calculated at baseline and after the experiment. The number of laps swam was recorded to adjust the metabolic rate according to the physical activity exertion (Ainsworth et al., 2011; Shono et al., 2001). For those who remained in the water without swimming, an increase of 25% in the resting metabolic rate was used to account for the involuntary movements (including shivering for thermogenesis) associated with staying put in the water. Detailed MET calculation is explained in Appendix A methods S2.

1.5. mRNA (messenger ribonucleic acid) processing and gene expression profiling

mRNA extraction was performed using the PAXgene Blood RNA Kit (Ref. 762174, Qiagen, Germany). Isolated RNA (ribonucleic acid) was quality controlled with an Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA) and quantified in a NanoDrop 1000 UV-Vis (Ultraviolet and visible spectrum 220–750 nm) Spectrophotometer (ThermoScientific, Wilmington, DE, USA). Microarray processing was performed in 2009 in four batches including 22, 20, 22 and 10 samples per day. Samples were processed in the order of arrival. Genome-wide expression analysis was performed using Illumina HumanHT-12 v3 Expression-BeadChip Kits. Two chips were processed in the first three batches adding one UHRR (Stratagene Universal Human Reference RNA, Agilent Technologies, Santa Clara, CA, USA) or one Ambion sample control per chip, and one chip was used for the last batch with two UHRR control samples in the same chip. Briefly, 200 ng of mRNA were reverse-transcribed into cRNA and biotin-UTP labeled using the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX, USA), and 1500 ng of cRNA were hybridized to the Illumina HumanHT-12v3 Expression-BeadChips (Illumina Inc., San Diego, CA, USA). Each Beadchip contains 12 microarrays allowing for parallel 12 sample processing. This chip interrogates about 25,000 genes with 49,895 probes using a single channel microarray based assay for whole transcriptome profiling. Hybridization, washing and staining steps were performed manually following strictly the manufacturer's standardized procedures described in the "Whole-Genome Gene Expression Direct Hybridization Assay Guide" (Illumina Part # 11286331 Rev. A). All the samples had a RNA Integrity Number (RIN) higher than 7.0 (Schroeder et al., 2006), an optical density-OD (A260/A280) ratio value >1.8 and an OD (A260/A230) ratio >1.5. Fluorescence signal was detected on an Illumina Beadarray scanner using Beadscan software (Illumina Inc., San Diego, CA, USA). After background subtraction, raw intensity data (idat) files were produced using Illumina GenomeStudio software. As a quality measurement, the signal to noise ratio P95/P05 derived by Genome Studio was used. We defined a minimum ratio of 2 to include the samples in further analyses.

1.6. Statistical analyses of exhaled THM levels

Continuous covariates were compared using *t*-test or Mann–Whitney rank-test. We tested the linear correlation between continuous variables using the Pearson's correlation test.

1.7. Bioinformatic preprocessing and statistical analysis of gene expression

Preprocessing of gene expression data were based on the Dunning et al. protocol (Dunning et al., 2007). Preprocessing included quality control of the data, filtering of probes, normalization, batch effects removal and transformation. From the initial pool of probes ($N = 49,895$) 319 unmapped probes were removed. The hybridization performance was compared with controls, and conducted a quality control of the array information. Probes with unknown or mismatched annotation were filtered. Phenotypic sex was corroborated using chromosome Y gene expression. An additional filtering step was performed removing all the potential cross reactivity (15,214 probes excluded) and polymorphisms encompassing probes reported in the literature (Ramasamy et al., 2013). From the remaining, 12 probes had missing values and were removed. Finally, we extended the filtering to additional Illumina reported polymorphisms or cross reacting probes removing 8327 more probes. Although only 26,023 probes were modeled, these probes included 24,772 genes, equivalent to 99.3% of the genes interrogated by the microarray. Expression

intensities were quantile normalized and log_2 transformed. This data matrix was submitted to Gene Expression Omnibus (GEO), and is available through GSE61225 accession number. Given that laboratory analyses were performed in several batches and they are subject to unintended technical variation, we used the "removeBatchEffect" function included in the limma package to reduce the noise of these technical variations on the data.

As the samples were paired (pre- post-exposure), they were analyzed assuming non-independency using paired *t*-tests and linear mixed models adjusting for subject as a random effect. In addition, the linear mixed model for the gene expression array was weighted for the array quality to reduce the nuisance of less reliable arrays and increase the power of our small sample estimates (Ritchie et al., 2006, 2015).

The absolute gain of THM exposure and metabolic rate were calculated by subtracting the pre-exposure from the post-exposure. Thus, a zero gain corresponds to the pre-exposure level (Appendix A Equation S4).

Models were fitted introducing one exposure at a time controlling for model stability and effect modification. In the random effect model the time invariant covariates (*e.g.* sex, or age) are included in the intercept and are not included as covariates in the models. Variance inflation factor (VIF) was used to test collinearity between the covariates. If any covariate showed a VIF >4 or the average mean VIF >2 for all the covariates the model was discarded. Beadarray and limma packages were used for statistical analyses using R v.3.3.1 (Dunning et al., 2007; R Core Team, 2016; Ritchie et al., 2015; Tingley et al., 2014). As the gene expression intensity is Log_2-transformed, we expressed our results as Log_2-Fold change (Log_2FC). All the gene expression comparisons are geometric mean changes in the Log_2 scale. For the mixed effects models the Log_2FC (equivalent to the geometric mean change in the beta coefficient of the model) per probe is reported in tables, and the equivalent fold change-FC (ratio change per unit) could be calculated by the reader. Downregulation of a gene corresponds to negative Log_2FC or FC <1 (ratio reduction per unit), while upregulation of a gene corresponds to positive Log_2FC or FC >1 (ratio increase per unit). Two different significant associations are reported: (1) the "nominal associations" (*p*-value <0.05) without correction for multiple comparison, and (2) the False Discovery Rate (FDR) (FDR <0.05) (Benjamini and Hochberg, 1995) or Q-value after correction for multiple comparisons.

Overexpression analyses were performed using the goana function of limma (Smyth, 2004). This function applies a hypergeometric Fisher test to evaluate if the differentially expressed genes are overrepresented in a gene universe tested (total genes analyzed). The overrepresentation test was split between those genes upregulated and those downregulated. We also explored The Molecular Signatures Database (MSigDB) using the Competitive Gene Set Test Accounting for Inter-gene Correlation (camera) function of limma and the database 5 (Gene Ontology curated genes), unlike the previous approach this gene set enrichment ranks all the probes uses all the probes interrogated by the microarray (independently of their statistical significance in the models).

2. Results

Study participants were 60% females with a mean age of 31 years (Standard deviation–(SD): 6.0), average weight of 71 kg (SD: 14), and average height of 171 cm (SD: 7.7) (Table 1). The resting metabolic rate were similar between groups (*p*-value = 0.7).

Table 1 – Description of the study population and samples collected.

Variable	
N (blood samples [*])	74
n (subjects)	33 [**]
Sex, female, *n* (%)	22 (59.5)
	Median [P25, P75]
Age (years)	30.2 [27.3, 33.6]
Weight (kg)	73.0 [62.0, 81.7]
Height (cm)	170 [166, 177]
Body mass index (kg/m^2)	23.6 [21.4, 26.9]
Body surface area (m^2)	1.8 [1.7, 2.0]
n (samples)	37 [***]
Physical activity and metabolic rate (40 min)	
Speed (meters/min)	21.9 [13.8, 25.0]
METs (baseline)	0.74 [0.71, 0.76]
METs (experiment)	2.28 [1.02, 3.31]
Water measurements (μg/L)	
Chloroform	15.9 [11.2, 18.6]
Bromodichloromethane	12.9 [10.9, 15.5]
Dibromochloromethane	11.6 [9.8, 14.2]
Bromoform	6.3 [5.3, 8.9]
Total brominated THM	31.1 [26.1, 36.8]
Total THM	47.4 [39.7, 55.1]
Air measurements ($\mu g/m^3$)	
Chloroform	29.9 [28.0, 34.7]
Bromodichloromethane	13.8 [11.5, 16.3]
Dibromochloromethane	13.0 [11.1, 15.4]
Bromoform	10.6 [8.0, 13.0]
Total brominated THM	36.9 [30.6, 43.1]
Total THM	68.8 [58.2, 73.0]
Exhaled air measurements pre-exposure ($\mu g/m^3$)	
Chloroform	0.76 [0.52, 1.02]
Bromodichloromethane	0.30 [0.24, 0.42]
Dibromochloromethane	0.16 [0.12, 0.28]
Bromoform	0.13 [0.07, 0.22]
Total brominated THM	0.54 [0.49, 0.93]
Total THM	1.37 [0.98, 1.79]
Exhaled air measurements post-exposure ($\mu g/m^3$)	
Chloroform	3.91 [1.94, 4.81]
Bromodichloromethane	1.58 [1.32, 1.81]
Dibromochloromethane	0.97 [0.64, 1.51]
Bromoform	0.37 [0.22, 0.53]
Total brominated THM	2.99 [2.31, 3.77]
Total THM	7.09 [4.60, 8.74]

METs (mL of oxygen consumption·kg/min) adjusted by basal metabolic rate using Harris–Benedict formula (see Appendix A methods 1).
THM: trihalomethanes; METs: metabolic equivalents.
[*] Every subject provided two measurements pre- and post-exposure. Four subjects repeated the experiment twice in different days and under different exercise conditions.
[**] Of the original 34 subjects one sample was mislabeled and the subject plus his two samples were discarded from the analyses).
[***] Number of samples collected per subject at baseline and after swimming in the pool. Total number of air and water pool samples (one per experiment).

Table 2 – Pearson correlations between metabolic rate and trihalomethanes measured in water, air and exhaled air. N = 37 (before–after pairs).

		METs		Water measurements (μg/L)						Air measurements (μg/m^3)						Exhaled air measurements post-exposure (μg/m^3)					
		Baseline	Post Experiment	TCM	BDCM	DBCM	TBM	Br-THM	TTHM	TCM	BDCM	DBCM	TBM	Br-THM	TTHM	TCM	BDCM	DBCM	TBM	Br-THM	TTHM
METs	Baseline	1.00																			
	Experiment	0.19	1.00																		
Water measurements (μg/L)	TCM	0.07	0.02	1.00																	
	BDCM	−0.05	−0.45	0.10	1.00																
	DBCM	−0.1	−0.43	−0.24	0.91*	1.00															
	TBM	−0.07	−0.32	−0.44	0.74*	0.93*	1.00														
	Br-THM	−0.08	−0.42	−0.19	0.93*	0.99*	0.93*	1.00													
	TTHM	−0.06	−0.42	0.11	0.98*	0.93*	0.81*	0.95*	1.00												
Air measurements (μg/m^3)	TCM	0.18	0.13	0.49	−0.16	−0.41	−0.44	−0.35	−0.21	1.00											
	BDCM	0.03	−0.03	−0.13	0.25	0.22	0.28	0.26	0.22	0.48	1.00										
	DBCM	0.05	−0.04	−0.37	0.36	0.42	0.50*	0.44	0.34	0.22	0.92*	1.00									
	TBM	0.12	−0.01	−0.06	0.40	0.43	0.55*	0.47	0.46	0.34	0.55*	0.61*	1.00								
	Br-THM	0.07	−0.03	−0.21	0.37	0.39	0.49	0.43	0.37	0.39	0.93*	0.95*	0.79*	1.00							
	TTHM	0.15	0.05	0.13	0.15	0.03	0.07	0.08	0.12	0.81*	0.87*	0.73*	0.7*	0.86*	1.00						
Exhaled air measurements post-exposure (μg/m^3)	TCM	0.06	0.47	0.31	−0.40	−0.49	−0.49	−0.48	−0.39	0.46	0.32	0.10	−0.11	0.13	0.34	1.00					
	BDCM	−0.05	0.42	0.09	−0.14	−0.16	−0.13	−0.15	−0.13	0.27	0.53*	0.39	0.10	0.39	0.40	0.85*	1.00				
	DBCM	−0.14	0.52*	−0.12	−0.22	−0.15	−0.09	−0.16	−0.20	0.07	0.37	0.36	0.04	0.30	0.23	0.76*	0.87*	1.00			
	TBM	−0.11	0.63*	0.08	−0.15	−0.11	−0.02	−0.10	−0.08	0.10	0.22	0.20	0.31	0.27	0.23	0.56*	0.69*	0.78*	1.00		
	Br-THM	−0.1	0.53*	0.00	−0.19	−0.16	−0.10	−0.16	−0.16	0.17	0.44	0.37	0.12	0.35	0.32	0.82*	0.96*	0.97*	0.83*	1.00	
	TTHM	0.0	0.52*	0.19	−0.33	−0.37	−0.35	−0.36	−0.31	0.36	0.39	0.22	−0.02	0.23	0.35	0.97*	0.94*	0.89*	0.70*	0.94*	1.00

TCM: Chloroform, BDCM: Bromodichloromethane, DBCM: Dibromochloromethane, TBM: Bromoform, Br-THM: Brominated trihalomethanes, TTHM: Total trihalomethanes.

* Significant correlations after Bonferroni correction.

The median level of total THM in water was 47.4 μg/L (IQR: 39.7–55.1). The median levels of water chloroform, bromodichloromethane and dibromochloromethane were similar (15.9, 12.9, 11.6 μg/L respectively), while bromoform was approximately half of the amount of the other compounds (median 6.3, IQR: 5.3–8.9), see Table 1. The Pearson linear correlation between the individual exhaled biomarkers to the environmental measurements and metabolic rate are shown in Table 2. There was a significant moderate positive correlation between the metabolic rate during the experiment and the post-exposure exhaled levels for all the THM except the chloroform and bromodichloromethane (see Table 2).

The main determinants of post-exposure exhaled THM levels were the concentration of THMs in the air of the swimming pool (*e.g.* the exhaled levels increased 0.09 μg/m^3 per 1 μg/m^3 of THM in the air of the swimming pool) and the metabolic rate (*e.g.* a mean increase of 0.94 μg/m^3 per 1 MET increase), see Table 3. There was no association between the pre- and the post-exposure exhaled THM levels. There was no relationship between THM water levels and the post-exposure exhaled levels for any of the THM, see Table 3.

A total of 1643 probes corresponding to 1436 genes were differently expressed after swimming (865 downregulated, 778 upregulated). We tested the differential expression before and after swimming in the pool and five potential exposures (Fig. 1): total THM (497 probes, 461 genes: 239 downregulated and 258 upregulated), total brominated THM (535 probes, 494 genes: 261 downregulated and 274 upregulated), chloroform (404 probes, 377 genes: 192 downregulated and 212 upregulated), bromoform (336 probes, 306 genes: 149 downregulated and 187 upregulated), and MET (2017 probes, 1711 genes: 920 downregulated and 1097 upregulated). There were 189 probes, 183 genes overlapping all the models (81 downregulated and 108 upregulated), Supplementary Table S5. When mutually adjusting any of the THM for the metabolic rate, the effect of the THM disappeared and there were 335 differentially expressed probes (297 genes) still related to MET. Of those only 63 probes (corresponding to 63 genes) overlapped with the other models. However, given that the metabolic rate is also related to the post-exposure exhaled THM levels (see Table 3), including both variables generated multicollinearity (VIF = 2.11 for the TTHM and MET). Multicollinearity reduces the power of the estimates and in some cases might produce wrong estimates and/or wrong directionality, and the results are difficult to interpret due to the power reduction. The observed Log_2FC were small per μg/m^3 increase for most of the biomarkers (range: −0.17 to 0.15) or the metabolic rate (range: −0.21 to 0.27). The biomarker showing biggest effect size was bromoform (Log_2FC range: −1.14 to 1.28), Appendix A Table S5.

The relationship between the 189 probes that overlapped between the models that were not mutually adjusted was explored. First, we explored potential common patterns of differential expression using an unsupervised hierarchical clustering. The cluster algorithm revealed two larger branches groups (Fig. 2): 23 measurements in pink (22 corresponding to post-exposure) showed statistically significant increases in both TTHM (p-value = 3.58E-06) and metabolic rate (p-value =9.53E-08). The second cluster (blue in the dendrogram) included 51 measurements (15 of them post-exposure), a gene expression pattern inversion (both up and down regulation of genes) compared to the first cluster was observed in most of these genes. When we compared only the samples after swimming in the pool, the first cluster showed a significant increase in the mean physical activity (p-value = 1.20E-03), but not significant differences in the total THM exhaled (p-value = 0.09).

Finally, we explored a Gene Ontology overrepresentation using the results of the model for exhaled total THM. In this model, among the 258 upregulated genes 11 pathways passed the FDR (Table 4), mostly related to cell signal transduction, and the last two to protein metabolic process downregulation and hematolymphoid organ development. A total of 29 pathways were associated with the 239 downregulated genes all related to mitochondria processes and several metabolic pathways (peptides, amides, organonitrogen and glycosyl biosynthesis) see Table 4. When restricting to the 189 common probes we did not obtain any significant association after FDR correction. Using a gene set enrichment approach (camera) we obtained a statistically significant, FDR < 0.05, list of 319 (out of 6166) Gene Ontology pathways. The enriched pathways were similar to those observed in the most focused overrepresentation analyses (Appendix A Table S6).

Table 3 – Factors determining the post-exposure exhaled THM levels. Change (beta and 95% confidence interval) in exhaled levels (μg/m^3) after swimming from a robust linear regression including levels in exhaled air before swimming, water and air, METs, sex, body surface area and age.

	Post-exposure exhaled breath levels					
	TCM β [95% CI]	BDCM β [95% CI]	DBCM β [95% CI]	TBM β [95% CI]	Br-THM β [95% CI]	TTHM β [95% CI]
Pre-exposure exhaled breath levels	−0.92 [−2.03,0.19]	−0.43 [−1.23,0.37]	−1.17 [−2.85,0.51]	0.09 [−0.6,0.77]	−0.72 [−1.62,0.19]	−0.95 [−1.79,-0.1]
Air levels	0.12 [0.03,0.21]	0.09 [0.03,0.14]	0.06 [0.001,0.12]	0.02 [−0.01,0.05]	0.07 [0.02,0.11]	0.09 [0.04,0.15]
Water levels	0.02 [−0.22,0.26]	−0.04 [−0.1,0.02]	−0.02 [−0.08,0.03]	−0.003 [−0.03,0.02]	−0.04 [−0.09,0.01]	−0.1 [−0.21,0.01]
MET	0.6 [0.1,1.1]	0.18 [0.03,0.33]	0.25 [0.06,0.44]	0.12 [0.03,0.2]	0.52 [0.15,0.9]	0.94 [0.2,1.69]
Males (*vs.* female)	−0.81 [−2.57,0.96]	−0.21 [−0.67,0.25]	−0.04 [−0.65,0.57]	0.04 [−0.2,0.28]	−0.21 [−1.43,1.01]	−1.42 [−4.46,1.62]
Body surface area	−0.23 [−5.5,5.03]	−0.15 [−1.24,0.94]	−0.45 [−2.08,1.18]	−0.24 [−0.74,0.27]	−0.87 [−4,2.25]	−0.87 [−8.37,6.64]
Age (years)	−0.03 [−0.15,0.08]	−0.03 [−0.08,0.01]	−0.03 [−0.07,0.01]	−0.01 [−0.02,0.004]	−0.07 [−0.16,0.02]	−0.14 [−0.37,0.09]

Notes: Every column represents a model per compound. The estimates were mutually adjusted for the covariates shown in the rows. Standard errors were robust adjusted. Pre-exposure exhaled air levels, air and water levels corresponds to the specific THM species tested in each column.

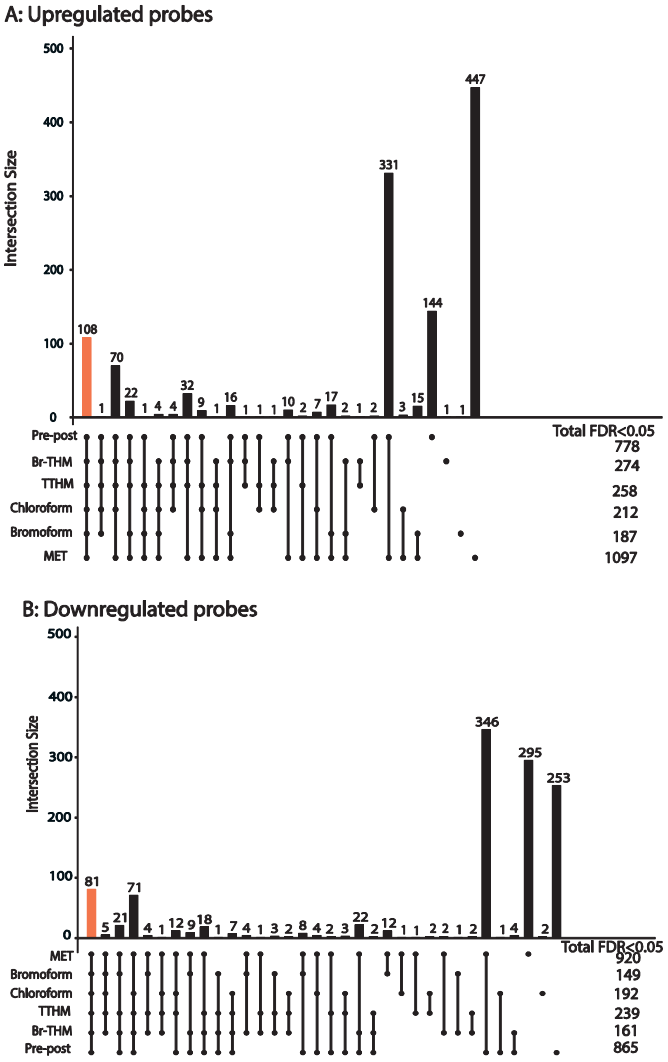

Fig. 1 – Bar diagram showing the overlapping probes with gene expression changes related to the swimming pool exposure, different trihalomethane exposures and metabolic rate after swimming in a chlorinated pool. Note: Panel A shows the upregulated probes, and Panel B the downregulated probes. The total probes evaluated were 26,023. Bars represent the number of probes (showed on top of the bar) that were statistically significant (FDR < 0.05) in the models marked with a dot below each bar. The overlap in different models is represented as connected dots. The orange bar shows the probes overlapping all the six models. On the bottom-right of each panel the total significant probes (FDR < 0.05) per model. FDR: False Discovery Rate.

3. Discussion

Using a pre- post-exposure experiment we observed an increase in the exhaled trihalomethane levels associated with environmental air levels and metabolic rate. We found several short-term gene expression changes related to the increase of exhaled THM levels and the metabolic rate. A total of 189 probes were identified in association with all the exhaled THMs and metabolic rate.

Epidemiological studies have linked the exposure to indoor pools environment with occupational asthma or airway irritation in pool workers and elite swimmers (Lévesque et al., 2006; Parrat et al., 2012). These associations have been attributed to airways irritants such as trichloramine, that is rapidly degraded in the environment and is not persistent *in vivo*. Trihalomethanes are volatile polar compounds abundant as a result of the water disinfection process, not known as airway irritants. However, trihalomethanes are absorbed through the airway, digestive tract and skin and may account for the global exposure to DBPs and are used as surrogates of total DBP exposure. In a previous report of this study, exhaled bromoform was associated with increased CC16 levels, a biomarker of epithelial lung permeability (Font-Ribera et al., 2010).

This is the first study in humans exploring the potential changes in gene expression associated with swimming in a chlorinated pool. Previously, only one experimental study evaluated the exposure of rat hepatocytes to a mixture of DBPs

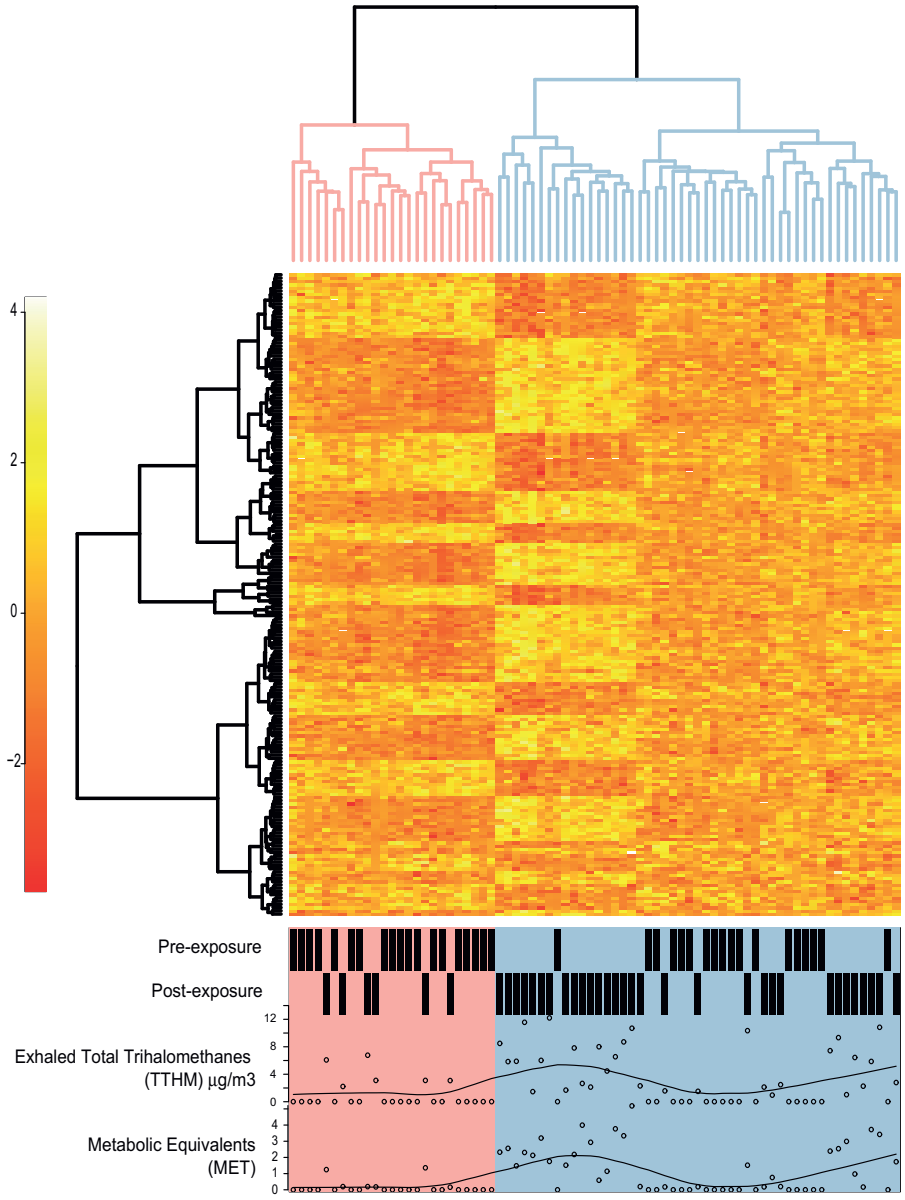

Fig. 2 – Cluster analysis of 74 RNA samples using 189 probes associated with both post-exposure exhaled air levels of the trihalomethanes and metabolic rate. Note: The heatmap shows differentially expressed genes that overlapped the different non-mutually adjusted models. Messenger RNA (mRNA) from whole blood of volunteers pre- and post-exposure to 40 minute swimming in a chlorinated pool. Each row represents a probe with columns representing each sample. The Log_2FC has been standardized and the color represents the changes in standard deviation respect the mean Log_2FC (z-scores). The top dendrogram shows the results of an unsupervised hierarchical clustering of 74 samples based on 189 genes, which separates two big clusters (in pink 22 samples after swimming and one before swimming showing both high levels of THM in exhaled breath and metabolic rate). In contrast, in blue 36 samples before swimming in the pool and 15 after swimming showing lower physical activity (and lower THM in exhaled breath) in the pool. In the bottom box, squares assigning the subject to pre- and post-exposure and experimental group. The absolute gain of exhaled Total trihalomethanes (TTHM) and metabolic equivalents (MET) in the middle box.

derived from ozonation/chlorination (Crosby et al., 2008). This study showed cytotoxicity due to the exposure to the mixtures at different concentrations, in the chlorinated most diluted concentration they observed an increase in the protein biosynthesis and a downregulation of the inflammatory response. In their study, higher concentrations of chlorine/ozone treated water develop cytotoxic tissue necrosis, while lower concentrations suggested a cathecolamine-like effect. The authors speculate that some of the volatile compounds may resemble endogenous catecholamines, able to induce downstream gene perturbation in G-coupled protein receptors. However, as in our experiment we do not have a control group not exposed to any of the

Table 4 – Overrepresented Gene Ontology pathways for the exhaled total THM change using the common differentially expressed genes (up and downregulated).

	Gene Ontology term	Ontology	N	DE	*p*-Value	FDR
Upregulated pathways						
GO:0007166	Cell surface receptor signaling pathway	BP	2352	34	1.29E-06	0.01
GO:0007165	Signal transduction	BP	5059	55	1.46E-06	0.01
GO:0044700	Single organism signaling	BP	5469	57	3.29E-06	0.01
GO:0023052	Signaling	BP	5476	57	3.44E-06	0.01
GO:0051716	Cellular response to stimulus	BP	6079	61	3.62E-06	0.01
GO:0007154	Cell communication	BP	5484	57	3.63E-06	0.01
GO:0035556	Intracellular signal transduction	BP	2373	33	4.70E-06	0.01
GO:0050896	Response to stimulus	BP	7237	68	5.25E-06	0.01
GO:0044754	Autolysosome	CC	8	3	1.33E-05	0.03
GO:0051248	Negative regulation of protein metabolic process	BP	941	18	2.11E-05	0.04
GO:0048534	Hematopoietic or lymphoid organ development	BP	689	15	2.57E-05	0.05
Down regulated pathways						
GO:0005739	Mitochondrion	CC	1456	28	7.15E-11	1.49E-06
GO:0005743	Mitochondrial inner membrane	CC	424	15	1.60E-09	1.67E-05
GO:0005740	Mitochondrial envelope	CC	626	17	6.20E-09	3.99E-05
GO:0019866	Organelle inner membrane	CC	476	15	7.69E-09	3.99E-05
GO:0007005	Mitochondrion organization	BP	563	16	9.83E-09	4.09E-05
GO:0031966	Mitochondrial membrane	CC	587	16	1.77E-08	5.32E-05
GO:0044429	Mitochondrial part	CC	855	19	1.79E-08	5.32E-05
GO:0032543	Mitochondrial translation	BP	112	8	6.86E-08	1.78E-04
GO:0070125	Mitochondrial translational elongation	BP	80	7	1.17E-07	2.65E-04
GO:0070126	Mitochondrial translational termination	BP	81	7	1.27E-07	2.65E-04
GO:0031967	Organelle envelope	CC	989	19	1.79E-07	3.25E-04
GO:0031975	Envelope	CC	992	19	1.88E-07	3.25E-04
GO:0006415	Translational termination	BP	94	7	3.56E-07	5.70E-04
GO:0033108	Mitochondrial respiratory chain complex assembly	BP	66	6	7.84E-07	1.16E-03
GO:0006414	Translational elongation	BP	121	7	1.98E-06	2.74E-03
GO:1901564	Organonitrogen compound metabolic process	BP	1948	25	2.97E-06	3.85E-03
GO:0005840	Ribosome	CC	204	8	6.53E-06	7.98E-03
GO:0043604	Amide biosynthetic process	BP	668	13	1.83E-05	0.02
GO:0030529	Intracellular ribonucleoprotein complex	CC	671	13	1.92E-05	0.02
GO:1990904	Ribonucleoprotein complex	CC	672	13	1.95E-05	0.02
GO:0031090	Organelle membrane	CC	2325	26	2.19E-05	0.02
GO:0006412	Translation	BP	581	12	2.23E-05	0.02
GO:0009119	Ribonucleoside metabolic process	BP	323	9	2.62E-05	0.02
GO:0043624	Cellular protein complex disassembly	BP	180	7	2.69E-05	0.02
GO:0043043	Peptide biosynthetic process	BP	603	12	3.21E-05	0.03
GO:0009116	Nucleoside metabolic process	BP	346	9	4.49E-05	0.04
GO:0043603	Cellular amide metabolic process	BP	856	14	5.75E-05	0.04
GO:1901566	Organonitrogen compound biosynthetic process	BP	1225	17	6.59E-05	0.05
GO:1901657	Glycosyl compound metabolic process	BP	366	9	6.92E-05	0.05

Notes: Ontologies are BP (Biological Process), CC (Cell Compound), N are the number of genes of universe (tested genes) present on the pathway, DE are the number of differentially expressed genes on the pathway.

trihalomethanes we cannot conclude that these mechanisms are also appearing in humans.

In addition, this is one of the first experimental studies in environmental epidemiology trying to characterize the short-term molecular events associated with swimming in a chlorinated pool. Previous studies have relied mostly on observational designs to characterize the events occurring in the people exposed to swimming pools. Some studies, also used some targeted biomarkers of effect such as cytokines and other inflammatory biomarkers. We are the first to use gene expression microarrays to comprehensively evaluate several disturbed molecular pathways measuring thousands of mRNA transcripts at the same time. Microarray technology had been used successfully in other environmental health research (Peretz et al., 2007; Wang et al., 2005; Wu et al., 2011). The use of this "omics" approach has been gaining popularity in epidemiological fields, despite some limitations in the interpretation of the results in observational studies (Vineis et al., 2009). The use of a before–after experimental design in our study increases the internal validity and it also reduces unintended variability through subject selection and paired analyses. The use of a cross-over subset in our sample also allowed to exclude potential carry-over effects of the pre-exposure THM exhaled levels and baseline gene expression. However, as we only measured two time points we cannot exclude other lagged gene expression changes that may appear

due to between subjects variability, or genes or pathways up or downregulated after the sample collection as observed in other environmental exposures (Wu et al., 2011).

A main limitation in this study is that we cannot disentangle the changes of gene expression associated with THM exposure from those associated with metabolic rate. Physical activity (including swimming) produces changes in gene expression ranging from oxidative stress and DNA (deoxyribonucleic acid) damage to antioxidative protective responses (Kruk and Duchnik, 2014). These responses are dependent of the physical activity intensity and of the oxygen consumption (metabolic rate): occasional and high endurance physical activity increases oxidative stress, while regular moderate intensity physical activity increases antioxidant activity. We observed that the post-exposure exhaled THM levels were related to the change in the metabolic rate. This association suggests that the metabolic rate change may not only be acting as a confounder but also it may act as an intermediate covariate in the causal pathway. The inclusion of an intermediate covariate for adjustment led to collinearity that precluded to distinguish the effect caused by the metabolic rate *versus* that caused by the exhaled THM change. Statistically, the use of an exploratory mediation analysis assumes a unidirectional causality link in which metabolic rate change increase the absorption and the levels of the toxicant (measured as post-exposure exhaled trihalomethane levels). This causal pathway might lead to gene expression changes partially or completely mediated through the intermediate covariate (Linden and Karlson, 2013). However, as we do not have a proper non-exposed control using these techniques could not help to disentangle the bias of the intermediate variable and could lead to artificial results. Future studies, should consider alternative designs and the use of control groups, *e.g.* including subjects swimming in waters with a minimal amount of DBPs (such as natural waters), include more cross-over samples and gene expression measured at different time lags, or standardize physical activity to reduce the variation between subjects.

In addition to a limited sample size, technical limitations of the microarrays should be considered. In mRNA microarrays, multiple probes may read different same gene transcripts which could result in inflated effect estimates and false positives (Wang et al., 2005). The mRNA stability is lower than that of DNA, the mRNA may decay if the sample processing and freezing is delayed after (Mathot et al., 2013). The number of samples run per chip is limited, in this study this implied four processing batches which may alter the results obtained even after batch correction (Johnson et al., 2007). The use of whole blood may limit the extrapolation of the information obtained to other tissues.

4. Conclusions

After swimming in a pool a set of 189 common probes showed differential expression. However, the magnitude of the change in the gene expression was minor, and its biological significance should be validated in bigger studies. Since the increase in exhaled trihalomethanes after swimming was correlated to the metabolic rate performed, it was not possible to properly disentangle the gene expression changes related to the DBP exposure.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2017.05.011.

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