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Short running head

Respiratory Biomarkers After Swimming in a Pool

Key words

Biomarkers, Clara cell secretory protein, disinfection by-products, exhaled breath condensate, fractional exhaled nitric oxide, respiratory health, swimming, swimming pools, trihalomethanes

List of Abbreviations and Definitions

CC16	16 KD Clara cell secretory protein
DBPs	Disinfection by-products
DPD	N,N-diethyl-p-phenylenediamine, colorimetric method to measure chloramines in water
EBC	Exhaled breath condensate
ELISA	Enzyme-linked immunosorbent assay
FeNO	Fractional concentration of orally exhaled nitric oxide
FEV1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
IFN- γ	Interferon gamma
IL-4	Interleukine 4
IL-8	Interleukine 8
IL-10	Interleukine 10
IL12p70	Interleukine 12p70
Ip10	Immune protein 10
IQR	Interquartile range
RANTES	Regulated upon activation, normal T-cell expressed and secreted
SD	Standard deviation
SP-D	Surfactant protein D
THMs	Trihalomethanes
TNF	Tumor necrosis factor

VEGF Vascular endothelial growth factor

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Outline of manuscript section headers

Abstract

Introduction

Materials and methods

Design

Respiratory biomarkers

Biomarkers of exposure

Environmental measurements

Other information collected

Statistical analysis

Results

Discussion

References

Tables 1-5

Figures 1-3

Abstract

BACKGROUND: Swimming in chlorinated pools involves exposure to disinfection by-products (DBPs) and has been associated with impaired respiratory health.

OBJECTIVES: We evaluated short-term changes in several respiratory biomarkers to explore mechanisms of potential lung damage related to swimming pool exposure.

METHODS: We measured lung function and biomarkers of airway inflammation (fractional exhaled nitric oxide –FeNO- and 8 cytokines and 1 growth factor (VEGF) in exhaled breath condensate), oxidative stress (8-isoprostane in exhaled breath condensate), and lung permeability (surfactant protein D -SPD- and the Clara cell secretory protein -CC16- in serum) in 48 healthy non-smoking adults before and after swimming for 40 min in a chlorinated indoor swimming pool. We measured trihalomethanes in exhaled breath as a marker of individual exposure to DBPs. Energy expenditure during swimming, atopy and CC16 genotype (rs3741240) was also determined.

RESULTS: Median serum CC16 levels increased from 6.01 to 6.21 $\mu\text{g/L}$ (average increase 3.3%, paired Wilcoxon test $p = 0.03$), regardless of atopic status and CC16 genotype. This increase was explained both by energy expenditure and different markers of DBP exposure in multivariate models. FeNO was unchanged overall but tended to decrease among atopics. We found no significant changes in lung function, SP-D, 8-isoprostane, 8 cytokines and VEGF.

CONCLUSIONS: A slight increase in serum CC16, a marker of lung epithelium permeability, was detected in healthy adults after swimming in an indoor chlorinated pool. Exercise and DBP exposure explained this association, without involving inflammatory mechanisms. Further research is needed to confirm the results, establish the clinical relevance of short-term serum CC16 changes, and evaluate the long-term health impacts.

Introduction

Swimming is practiced extensively in western countries (Vaz et al. 1999). Despite the benefits of physical activity, health concerns are growing because swimming in pools involves exposure to disinfectants and disinfection by-products (DBPs), such as trihalomethanes, one of the classes of DBPs at highest concentration in swimming pools, and trichloramine, a known irritant (WHO 2006). A range of acute symptoms has been described among bathers after accidental exposure to high levels of chlorine in swimming pools, including mucosal and ocular irritation, cough, rash, dyspnea, and lung-function decline (Bonetto et al. 2006; Grasemann et al. 2007). Subjects exposed chronically to the swimming pool environment, such as pool workers, showed irritant eye, nasal, and throat symptoms (Jacobs et al. 2007; Massin et al. 1998). Cases of occupational asthma and trichloramine sensitization have been described in pool lifeguards (Thickett et al. 2002). Although an increased asthma risk among children attending pools has been suggested but not confirmed (Goodman and Hays 2008; Font-Ribera et al. 2009), respiratory symptoms and asthma among competitive swimmers are consistently more prevalent compared to other athletes (Goodman and Hays 2008). However, one of the unsolved questions is what are the biological mechanisms behind these health effects (Bonetto et al. 2006; Grasemann et al. 2007).

The development of methods to evaluate respiratory and systemic biomarkers in blood, exhaled breath condensate (EBC) and exhaled breath has allowed the assessment of pathobiological mechanisms underlying respiratory disorders (Bonetto et al. 2006) and the detection of early subclinical respiratory effects after acute or chronic environmental exposures, including swimming pool attendance. Lung surfactant proteins, such as Clara cell secretory protein (CC16) or Surfactant protein D (SP-D), are secreted in the lung epithelium and move passively across the epithelial barrier into the serum down a strong gradient (Broeckaert et al. 2000). A change in the concentration of lung surfactant proteins in serum

has been proposed as a marker to detect early permeability changes in the lung epithelium (Broeckaert et al. 2000). Fractional concentrations of orally exhaled nitric oxide (FeNO) is a marker of eosinophilic airway inflammation (Choi et al. 2006) and has been shown to increase after short-term exposure to mould (Stark et al. 2005). Soluble molecules can be detected in EBC, including pro-inflammatory cytokines, growth factors and oxidative stress biomarkers, and have been used to monitor different aspects of diseases such as asthma or chronic obstructive pulmonary disease, as well as the effects of environmental stressors or physical exercise (Bonsignore et al. 2003; Carbonnelle et al. 2002, 2008; Massin et al. 1998; Nanson et al. 2001).

Proposed mechanisms of respiratory damage related to swimming-pool exposure include airway inflammation (Bonetto et al. 2006; Grasemann et al. 2007; Moreira et al. 2008; Pedersen et al. 2009), oxidative stress (Varraso et al. 2002), and hyperpermeability of the lung epithelium (Bonetto et al. 2006; Carbonnelle et al. 2002, 2008). Increased permeability of the lung epithelium has been evaluated extensively, and some authors suggest that it may result in increased airway inflammation and higher risk of sensitization and allergic diseases (Bernard 2007). The role of previous atopic status is unclear because some studies have found higher asthma risk for swimming pool attendance among atopics (Bernard et al. 2006, 2007, 2008), but another has not (Font-Ribera et al. 2009). Few studies have measured lung function and respiratory biomarkers after swimming in chlorinated pools (Carbonnelle et al. 2002, 2008; Moreira et al. 2008; Pedersen et al. 2009). Lung function and FeNO were consistently unaltered in the studies, but contradictory results were obtained for lung epithelium permeability, estimated with serum levels of surfactant proteins. Consequently, the evidence remains inconclusive and inconsistent for some biomarkers too.

The aim of this study was to explore short-term respiratory changes in healthy adults after swimming in an indoor chlorinated swimming pool by measuring lung function and a wide

range of biomarkers that may reflect different mechanisms of effect, specifically, airway inflammation (FeNO and 8 cytokines and a growth factor in EBC), oxidative stress (8-isoprostane in EBC), and epithelial lung permeability (SP-D and CC16 in serum), taking into account both exposure to DBPs and physical exercise. Associations between swimming and these outcomes may provide clues regarding potential mechanisms through which swimming-related exposures might affect respiratory health.

Materials and Methods

Design

The study has a cross-over design involving 50 non-smoking adults who were recruited through open advertisements on the Internet and at local universities. A screening questionnaire was used to verify eligibility among subjects (non-smoking adults, 18 to 50 years old, without respiratory diseases such as ever asthma or having had a cold in the last 3 weeks). Participants were requested to avoid swimming pools during the week before the session and taking a shower the day of the swimming experiment. The study was approved by the ethics committee of the research centre following the international regulations, and all volunteers signed an informed consent before participation.

A single, indoor, 25-m long chlorinated swimming pool in Barcelona, Spain, was used for the study. Every day, 1 to 4 participants were evaluated between 9:00 and 14:00 h (before lunch) in May, June, September, or October 2007. Before and after swimming in the chlorinated pool for 40 min, a battery of measurements and biological samples were collected to evaluate respiratory biomarkers according to a strict schedule (Figure 1). Biological samples and measurements before and after swimming were obtained in a room inside the sports center where the swimming pool was located, but separated from the swimming pool area.

Respiratory biomarkers

8-isoprostane and cytokines. Exhaled breath condensate (EBC) was obtained approximately 70 min before swimming began and 35 min after swimming ended using an EcoScreen[®] condenser (Jaeger GmbH, Wirzburg, Germany) following the ATS/ERS Task Force recommendations (Horvath et al. 2005). Samples were obtained through breathing at normal frequency and tidal volume until a total expiratory volume of 180 L was achieved.

After collection, the condensing device was centrifuged at 4°C, and the resultant total EBC volume (approximately 4 mL) was transferred into Eppendorf tubes and rapidly frozen in liquid nitrogen. All samples were lyophilized and stored at –80°C before analysis. 8-Isoprostane was analyzed through an enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical, Ann Arbor, MI, USA). The following 8 cytokines and a growth factor were determined: Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES), vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF), interleukin (IL)12p70, IL-4, IL-8, IL-10, interferon gamma (IFN- γ) and IFN- γ -induced protein 10 (Ip10) using the BD™ Cytometric Bead Array (CBA), and the BD FACSCalibur™ Flow Cytometer, a particle-based immunoassay. Levels were characterized as pg/mL of EBC.

Clara cell secretory protein (CC16) and surfactant pneumoprotein D (SP-D). Two 5-mL vacutainer serum tubes were collected from each participant by venipuncture before swimming and 70 min after swimming. Samples were centrifuged at 2500 rpm for 15 min, and serum was subsequently distributed in 1.8-mL aliquots, and stored at -80 °C. CC16 and SP-D were analyzed with an ELISA, using commercial kits (Biovendor Laboratorní medicína a.s., Modrice, Czech Republic). Intra-assay and inter-assay coefficients of variation ranged from 2.0% to 2.5% in both cases for serum SP-D, and from 4.0% to 5.0% also in both cases for serum CC16. The minimum detectable concentration in serum was set to be 0.2 ng/mL for SP-D and 20 pg/mL for CC16 (Biovendor Laboratorní medicína a.s.). Levels were expressed as $\mu\text{g/L}$ of serum.

Fractional concentration of orally exhaled nitric oxide (FeNO) was measured 40 min before and 80 min after swimming with an electrochemical portable device (NIOX-MINO™, Aerocrine, Solna, Sweden), with a constant airflow rate of 50 mL/s. Duplicate measurement was performed in 50% of the participants to evaluate reproducibility, resulting in a coefficient of variation of 9.7% (SD=10.6). Levels were expressed as parts per billion (ppb).

Lung function. Forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC) were measured 30 min before swimming and 60 min after swimming, with an EasyOne® portable spirometer (NDD, Medical Technologies, Zürich, Switzerland) following standard recommendations (Miller et al. 2005). FEV-1 and FVC were expressed as the percentage from the predicted value by age, gender, and height (Roca et al. 1986).

Biomarkers of exposure

The four trihalomethanes (THMs), chloroform, bromodichloromethane, dibromochloromethane, and bromoform, were measured in exhaled breath before entering the swimming pool (80 min before swimming) and just after swimming (5 min after leaving the pool) (figure 1), as markers of individual exposure to DBPs. Exhaled breath samples were collected using a portable system for end-exhaled breath sampling, which has been described previously (Lourencetti et al. 2010). Briefly, volunteers were required to breathe through the sampling device equipped with an adsorption cartridge packed with Tenax TA. A total volume of 1 L was collected per person. The air passed through a stainless-steel cartridge (0.5-cm diameter and 9-cm long) containing 1.8 g of Tenax TA (60/80 mesh). Chloroform, bromodichloromethane, dibromochloromethane, and bromoform were determined by an Automatic Thermal Desorption System (ATD 400, Perkin-Elmer) coupled to an Autosystem gas chromatograph with electron capture detection (GC-ECD, Perkin-Elmer). Concentrations were expressed as $\mu\text{g}/\text{m}^3$.

Environmental measurements

Environmental measurements were done to characterize the swimming pool and to complement the exposure assessment to DBP. Free chlorine, THMs and mono-, di-, and trichloramine were measured in pool water. A 1-L composite water sample was collected at four different points of the pool for each participant while he or she was swimming. A single value for free chlorine, monochloramine (NH_2Cl), dichloramine (NHCl_2), and trichloramine

(NCl_3) was obtained for each participant as measured by DPD procedure with a portable photometer (DINKO Inst., Inc). The methods for water and air THM analyses have been described elsewhere (Lourencetti et al., 2010). Briefly, for THM analyses, 5 mg of sodium thiosulfate was added to a 40-mL glass vial with screw-cap and PTFE-lined silicone septa. Water samples were stored at 4 °C until laboratory analysis on the same day. Trihalomethanes in water were determined using a SOLATek 72 multi-matrix vial autosampler coupled to a purge-and-trap concentrator (Tekmar 3100), which transfers the sample directly to a GC coupled to a mass spectrometer (Voyager MS, ThermoQuest Finnigan, USA) and had a coefficients of variation between 0.98% and 5.6%. An indoor air sample for THM measurements was collected for each participant with a pump located 60 cm above the floor and 1.5 m from the pool border, at 7 mL/min flow rate for 20 min through an adsorption cartridge filled Tenax TA. Quality control was assured by daily calibration of the pump. The four THMs were measured as described for exhaled breath samples and were expressed as $\mu\text{g}/\text{m}^3$.

Additional air samples were collected to measure trichloramine in a subset of the days (6 days). Air was collected with a constant flow sampling pump (flow rate of 1.2 L/min for an average of 115 min, standard deviation (SD) = 32), within 1 metre 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 the Utrecht University following the method described by (Hery et al. 1995); further details are available elsewhere (Jacobs et al. 2007). Trichloramine measurements were used for comparison with other swimming pools, but were not used as personal exposure estimates because only 2 out of the 6 days with trichloramine levels coincided with participants (two subjects).

Other information collected

Questionnaires were used to collect information on personal and family history of atopic diseases, exposure to environmental tobacco smoke, diet, sociodemographic data, frequency and duration of swimming pool attendance and other physical activity and way of commuting to the swimming pool facility. Weight and height were measured with standard procedures. Exercise intensity during swimming was estimated using the distance swum by each participant during the 40 min. Energy expenditure (Kcal) was estimated using the swimming speed and the weight of the participant, assuming that swimming at 46 m/min equals 11 metabolic equivalent tasks (METs) (Kcal/kg/h):

$$\text{Kcal} = \text{weight (kg)} * \text{speed (m/min)} * 40 \text{ min} * 1 \text{ h}/60 \text{ min} * 11 \text{ (Kcal/kg/h)} / 46 \text{ (m/min)}$$

(Ainsworth et al. 2000). Atopic status was measured with the Phadiatop test, a qualitative test for serum-specific IgE to a mixture of common allergens (Vidal et al. 2005). A single nucleotide polymorphism (SNP) in the *CC16* gene (rs3741240), known to modify gene expression, was genotyped using Sequenon (CEGEN-Santiago). DNA was extracted from peripheral blood samples.

Statistical analysis

The distribution of each biomarker was evaluated with a test for normality evaluating skewness and kurtosis. Mean or median values were reported accordingly to describe central tendencies. We calculated the individual change in the concentration of each biomarker after swimming in the pool (post – pre concentration). Samples with cytokines under the detection limit before (30.1%) or after (27.1%) swimming were imputed half the detection limit. Those with undetectable levels before and after swimming were excluded from the statistical analysis. A non-parametric test was used to evaluate if there was a significant change in the concentration of each biomarker. Linear regression models were fitted to calculate the association between changes in the concentration of each respiratory biomarker and the personal markers of DBP exposure and exercise intensity. The beta coefficient of a change in

a unit in the concentration of each respiratory marker was calculated for an increase from quartile 25 to quartile 75 in the exposure parameters. All covariates were tested in each model, and only those that were statistically significant were retained in the multivariate models. The *p*-value threshold for statistical significance was set up at <0.05 . Interactions were tested by introducing the product of the variables in the regression model. All the statistical analyses were performed with the statistical package STATA 8.2.

Results

Fifty subjects were recruited for the study. Two subjects with history of asthma were excluded for the current analysis, resulting in a sample of 48 subjects. Most participants were women (65%), highly educated (92% with university studies) with an average age of 30 years (SD = 6.1) and 30% were positive to the Phadiatop test. Twenty percent were regular swimmers (at least once per month) and 54% practiced sport at least once a week. Regarding the *CC16* genotype (A38G), frequencies were 39%, 12%, and 49% for AG, AA, and GG, respectively. The genotyping frequency was 91%, and it was in Hardy-Weinberg equilibrium (HWE, $p = 0.365$). Minor allele frequency (MAF) was similar to the ones described in the International HapMap project for European individuals (The International HapMap Consortium 2003). The mean speed during swimming was 22.5 m/min (SD = 9.7), and the mean energy expenditure was 248.5 Kcal (SD = 120.6). We had one missing value for energy expenditure, one for THM in exhaled breath, one for FeNO and 3 for 8-isoprostane in EBC. Average free chlorine level in the pool water was 1.17 mg/L (SD = 0.4). Average total THM concentrations in water was 45.4 $\mu\text{g/L}$ (SD = 7.3) (Table 1). The average (SD) level of THM in exhaled breath before swimming in $\mu\text{g/m}^3$ was 1.19 (0.40) for total THMs, 0.72 (0.28) for chloroform, 0.25 (0.09) for bromodichloromethane, 0.13 (0.06) for dibromochloromethane, and 0.10 (0.07) for bromoform. After swimming, THMs in exhaled breath increased on

average about 7 times. The increase was similar by age group, sex, or body mass index (results not shown). Chloroform levels in exhaled breath were significantly correlated with levels in the swimming pool's air, but not with levels in water (Table 2). Dichloramine in water was inversely and significantly correlated to brominated THMs but not to chloroform in water, air and exhaled breath. Free chlorine in water was not significantly correlated to total THMs in water, whereas it was significantly correlated to total THMs in air and exhaled breath. The energy expenditure correlated significantly only with bromoform concentration in exhaled breath after swimming. Trichloramine in water was undetectable, and monochloramine correlated with the same DBPs as dichloramine, so only the last is shown in the tables.

The concentration of CC16 in serum was increased significantly after swimming, with an overall median increase of 0.47 $\mu\text{g/L}$ (3.3% increase) (Table 3). No significant changes were detected in % predicted FEV1, %predicted FVC, FEV1/FVC, FeNO, serum SP-D, 8-isoprostane (Table 3), or cytokines in EBC (Table 4). The increase in serum CC16 concentration was significantly correlated with different indicators of DBP exposure (negatively with dichloramine in water and positively with free chlorine in water and bromodichloromethane, dibromochloromethane and bromoform in exhaled breath) and with energy expenditure (Table 3 and Figure 2). In multivariate models, both energy expenditure and markers of DBP exposure remained significantly associated with the increase in CC16 after mutual adjustment (Table 5). An interquartile range (IQR) increase in energy expenditure was associated with a significant increase in 8-isoprostane in EBC after swimming. An interaction was found with the change in 8-isoprostane and swimming regularly (p -value = 0.04). 8-Isoprostane decreased among those who swam regularly (median change -1.0 pg/mL ; $\text{SD} = 1.2$; p -value = 0.04), whereas it tended to increase among those who did not swim regularly (0.62 pg/mL ; $\text{SD} = 2.1$; p -value = 0.09).

When the change in the biomarker concentration was calculated as a relative measure (post-pre/pre levels), the same patterns were found. Bivariate analyses showed that the changes in the levels of these respiratory biomarkers did not differ by sex, age, body mass index or the time spent in active commuting (walking or cycling) to the swimming pool facility. Atopic participants had higher baseline FeNO concentrations than non-atopic participants, and they tended to have a decrease in FeNO after swimming, whereas non-atopic subjects remained stable (Figure 3). The increase in CC16 concentration in serum was not modified significantly by atopic status. Furthermore, CC16 change was not different among CC16 genotypes, modeled as dichotomous (GG vs. AA/AG) (p -value = 0.507)(results not shown).

Discussion

A slight but significant increase in lung epithelial permeability was detected, as estimated by serum CC16, in healthy adult volunteers after swimming in a chlorinated pool. Energy expenditure during swimming and change in THM concentrations in exhaled breath after swimming (indicating higher DBP exposures) were significant predictors of increases in serum CC16, suggesting that these exposures may have contributed to an increase in lung permeability. Significant changes in lung function tests or markers of inflammation or oxidative stress were not observed in adults after swimming in a chlorinated pool.

The lack of an association between swimming and lung function and FeNO was consistent with previous studies with a comparable design (Carbonnelle et al. 2002, 2008; Moreira et al. 2008; Pedersen et al. 2009). However, evidence on serum CC16 is less consistent. Serum CC16 did not vary significantly in 11 young adults (Carbonnelle et al. 2008) and in 16 children (Carbonnelle et al. 2002), whereas it decreased (29% decrease) among 13 adults after swimming in a chlorinated pool, with a concentration of trichloramine

in air between 160 and 280 $\mu\text{g}/\text{m}^3$ in the first study (Carbonnelle et al. 2008) and of 490 $\mu\text{g}/\text{m}^3$ in the second (Carbonnelle et al. 2002). The study by Carbonnelle et al. detected an increase in serum CC16 levels among 14 elite swimmers after swimming in a chlorinated pool (44% increase) and a non-chlorinated pool (52% increase) (Carbonnelle et al. 2002), suggesting that the hyperpermeability of the lung epithelium after swimming could be caused by physical activity (Nanson et al. 2001). We showed in the present study that the both exercise intensity during swimming and markers of DBP exposure were associated with the increase in serum CC16 after mutual adjustment, supporting the hypothesis of independent effects of exercise and chemical exposure on the permeability of the lung epithelium.

The unchanged concentration of 8-isoprostane after swimming suggests the lack of association with oxidative stress in the airways.. However, 8-isoprostane tended to increase with energy expenditure, in accordance with a previous study showing that oxidative stress increases after physical exercise in healthy subjects (Moller et al. 1996). No changes were detected in the eight cytokines and one growth factor measured in EBC, in accordance with a previous study that did not find changes in other markers of inflammation after swimming in a chlorinated pool in 21 adolescents (Pedersen et al. 2009). Although the concentrations of cytokines in EBC of our healthy study population were relatively low (about 1 pg/mL), they were detectable in the large majority of samples (about 80%). However, partly because of the lack of appropriate reference values, the validity of using these proteins in EBC as markers of acute inflammation in healthy subjects needs to be determined.

The present study has a larger sample size than previous studies with a similar design (N=48, compared to 30 (Moreira et al. 2008) 29 (Carbonnelle et al. 2002), 21 (Pedersen et al. 2009), and 11 (Carbonnelle et al. 2008) . However, statistical power could still be limited for detecting minor changes in some biomarkers with statistical significance. The timing of sample collection was selected to recognize the specific expression dynamics of the different

biomarkers and was highly controlled during fieldwork. However, available data on expression dynamics of some biomarkers were limited or inconsistent. For example, an increase in FENO has been observed right after swimming (Carbonnelle 2002) and also 6 h after mould exposure (Stark 2005). Therefore, undetected changes in some biomarkers due to inappropriate sample timing cannot be ruled out. Although THMs are not irritants and are not likely the putative agents for the respiratory effects associated to the swimming pool environment, their occurrence in exhaled breath was used as a surrogate for DBP dose because THMs are the most prevalent DBPs in swimming pools and are easy to measure in exhaled breath. The observed dichloramine concentrations (0.43 mg/L; Table 1) in water were low in comparison to other swimming pools using chlorine for disinfection (Weaver et al., 2009). Furthermore, the same DPD method was used for all participants; therefore, the influence of the biases should be minimal. The season when the study was conducted (spring-summer) probably represented lower levels of DBP exposures than the rest of the year because the facility was highly ventilated with doors and windows opened. Trichloramine in the air ranged from 0.17 to 0.43 mg/m³ (mean of 0.29 mg/m³), which is below the World Health Organization recommendations of 0.5 mg/m³ (WHO 2006), but comparable to the study by Carbonnelle et al. 2008.

The measurement of a battery of respiratory biomarkers allowed us to explore short-term respiratory changes that may reflect different mechanisms of respiratory effect in relation to swimming pool exposure. However, there is limited knowledge of the clinical significance to interpret the health impacts of the biomarkers measured. The present study replicates with a higher sample size the methods of previous studies and provides new pieces of evidence on biomarkers not measured previously, including markers in exhaled breath condensate. It suggests for the first time that atopic status and *CC16* genotype do not modify the effect of swimming in a pool on the permeability of the lung epithelium. Furthermore, we

attempted to disentangle the effects of chemical exposure and exercise by measuring individual exposure to DBPs and energy expenditure during swimming.

The increase in serum CC16 after swimming in a well-maintained and highly ventilated indoor swimming pool confirms the high sensitivity of this assay to detect subtle changes in the concentration of this biomarker after environmental exposures. The fact that no differences in serum CC16 levels were detected by the *CC16* genotype further supports the hypothesis that the association is due to an increased permeability of the lung epithelium rather than an increase in CC16 synthesis, which may differ by genotypes (Laing et al. 2000). The higher molecular weight of SP-D (130 KDa) (Kishore et al. 2006) compared to CC16 (16 KDa) (Broeckaert et al. 2000) probably explains the lack of increase in serum concentration of this protein after swimming because its higher molecular weight would not permit the passive diffusion of the molecule through the epithelium barrier. Previous studies that measured other surfactant proteins (SP-A and SP-B) in serum in similar settings found inconsistent results (Carbonnelle et al. 2002, 2008).

We assessed the role of atopy as an effect modifier because some epidemiological studies have reported an increased asthma risk for swimming pool attendance among atopic children (Bernard *et al.* 2006, 2007, 2008, 2009). Baseline serum CC16 levels and the change after swimming were similar among atopics and non-atopics. Atopic status did not modify the effect of swimming pool exposure on the markers studied nor in pulmonary function, in agreement with a previous study (Bonetto et al. 2006). However, atopic participants had higher baseline FeNO levels, and atopy modified the effect of swimming (p -value = 0.02). FeNO remained unchanged among non-atopics, whereas it tended to decrease among atopics. Moreira et al. found no changes on FeNO that did not vary by atopic status or asthma in 30 competitive swimmers after a training session.

Among the battery of respiratory biomarkers evaluated, only serum CC16 levels changed significantly after swimming. Given the moderate increase detected (3.3%), the high variability in CC16 levels in healthy subjects (Broeckaert et al. 2000), and the lack of reference values of CC16, the clinical relevance of this short-term effect is unclear (Carbonnelle et al. 2008) and further studies are necessary to establish the health impacts of short-term serum CC16 changes (Broeckaert et al. 2000; Lakind et al. 2007). Further, previous studies have shown that this acute increase in serum CC16 is transient and that serum CC16 returns to baseline levels a few hours after exposure ceases (Carbonnelle et al. 2002, 2008). We interpret the increase in lung epithelial permeability after swimming as an acute physiologic reaction of the lung caused by exercise and exposure to some DBPs. Long-term effects cannot be extrapolated from these results until the clinical and physiological relevance of CC16 short-term change is understood further.

In summary, we detected a slight increase in serum CC16, which is a marker of lung epithelium permeability, in healthy adults after 40 min of swimming in an indoor chlorinated pool. Exercise and DBP exposure explained this association, without involving further inflammatory mechanisms. Further research is needed to confirm the results, disentangle the effects of exercise and DBP exposure, establish the clinical relevance of short-term serum CC16 changes, and evaluate the long-term respiratory health impacts of swimming.

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Table 1. Physico-chemical parameters in water, air, and exhaled breath and exercise intensity performed by participants (n = 48)

	Measurement	Mean (SD)	Median	Min	Max
Water					
	Free chlorine (mg/L)	1.17 (0.4)	1.10	0.5	2.17
	NHCl ₂ (mg/L)	0.43 (0.1)	0.46	0.16	0.65
	Temperature (°C)	27.2 (0.4)	27.4	26.5	27.7
	pH	7.3 (0.1)	7.3	6.9	7.5
	CHCl ₃ (µg/L)	16.1 (3.4)	16.7	8.5	20.8
	CHCl ₂ Br (µg/L)	12.3 (2.3)	11.9	9.3	22.8
	CHClBr ₂ (µg/L)	10.9 (3.1)	10.5	6.5	22.6
	CHBr ₃ (µg/L)	6.1 (2.4)	5.7	3.0	16.2
	Total THMs (µg/L)	45.4 (7.3)	45.5	35.2	75.2
Air					
	CHCl ₃ (µg/m ³)	35.0 (12.3)	31.4	19.5	61.6
	CHCl ₂ Br (µg/m ³)	14.6 (5.0)	13.0	7.5	23.4
	CHClBr ₂ (µg/m ³)	13.2 (4.3)	12.4	6.0	26.2
	CHBr ₃ (µg/m ³)	11.2 (5.2)	8.4	4.4	22.6
	Total THMs (µg/m ³)	74.1 (23.7)	68.9	44.0	124.9
Exhaled breath (after swimming) ^a					
	CHCl ₃ (µg/m ³)	4.5 (1.7)	4.6	1.1	8.1
	CHCl ₂ Br (µg/m ³)	1.8 (0.5)	1.6	0.7	3.2
	CHClBr ₂ (µg/m ³)	1.2 (0.5)	1.2	0.3	2.8
	CHBr ₃ (µg/m ³)	0.5 (0.2)	0.4	0.1	1.3
	Total THMs (µg/m ³)	7.9 (2.8)	7.7	2.3	14.0
Exercise intensity ^a					
	Distance swam (km)	0.90 (0.4)	0.95	0.05	1.75
	Energy expenditure (Kcal)	248.5 (120.6)	241.9	16.8	603.3

SD: standard deviation. NHCl₂: dichloramine; CHCl₃: chloroform; CHCl₂Br: bromodichloromethane; CHClBr₂: dibromochloromethane; CHBr₃: bromoform; THMs: trihalomethanes.

^an = 47.

Table 2. Spearman correlation coefficients (r) between the different exposure indicators measured (n =47)

Medium (concentration)	Water (µg/L)		Concentration in exhaled breath after swimming (µg/m ³)					Energy expenditure (Kcal) ^a	
	Free Cl	NHCl ₂	CHCl ₃	CHCl ₂ Br	CHClBr ₂	CHBr ₃	TTHMs		
Water (µg/L)	Free Cl		0.24	0.34*	0.48*	0.27	0.35*	0.16	
	NHCl ₂	-0.28		-0.22	-0.44*	-0.44 *	-0.50*	-0.37*	-0.26
	CHCl ₃	-0.24	0.17	0.27	-0.03	-0.32	0.01	0.07	-0.04
	CHBr ₃	-0.03	-0.31*	-0.40*	-0.05	0.25	0.24	-0.17	0.08
	TTHMs	-0.04	-0.40*	0.06	0.19	0.18	0.48*	0.14	0.04
Air (µg/m ³)	CHCl ₃	0.19	0.04	0.64*	0.40*	0.13	0.29*	0.52*	-0.001
	CHBr ₃	0.24	-0.29*	0.22	0.35*	0.29	0.55*	0.30*	0.12
	TTHMs	0.38*	-0.13	0.55*	0.48*	0.34*	0.43*	0.53*	0.03
Exhaled breath after (µg/m ³)	CHCl ₃				0.83*	0.60*	0.55*	0.94*	0.14
	CHCl ₂ Br					0.80*	0.72*	0.94*	0.18
	CHClBr ₂						0.74*	0.79*	0.18
	CHBr ₃							0.70*	0.32*
	TTHMs								0.19

NHCl₂: dichloramine; CHCl₃: chloroform; CHCl₂Br: bromodichloromethane; CHClBr₂: dibromochloromethane; CHBr₃: bromoform; TTHMs: total trihalomethanes.

*p-value < 0.05.

^aKcal: kilocalories expended during the 40 min.

Table 3. Level of respiratory markers before and after swimming. Linear regression coefficients of the change after swimming for the exposure parameters

	N	% predicted FEV1	% predicted FVC	FEV1/ FVC	FeNO (ppb)	8-isoprostane (pg/mL)	SP-D (µg/L)	CC16 (µg/L)
Before. Median	48	97.3	98.1	0.83	13	1.6	54.4	6.01
(IQR)		(90.1,103.6)	(90.0,105.4)	(0.8,0.9)	(10.5,18.5)	(1.0,2.0)	(39.3,68.0)	(3.9,7.7)
After. Median	48	96.3	95.9	0.83	12.5	1.3	55.1	6.21
(IQR)		(90.4,105.1)	(90.7,104.2)	(0.8,0.9)	(10,17.5)	(0.6,2.5)	(45.4,85.0)	(4.6,8.4)
Change. Median		-0.6	-2.0	0.0	0.0	-0.03	1.0	0.47
(IQR)		(-2.5,2.4)	(-5.1,3.9)	(-0.02,0.04)	(-2,2)	(-0.8,1.1)	(-3.7,6.6)	(-0.3,1.1)
p-value (change≠ 0) ^a		0.83	0.46	0.24	1.00	0.91	0.44	0.03
Change in respiratory markers for an increase from quartile 25 to quartile 75 in exposure parameters (95% Confidence interval) ^b .								
Free chlorine water (mg/L)		0.54 (-0.90, 1.99)	-2.44 (-5.77,0.88)	0.02 (0.00-0.04)*	-1.22 (-2.51,0.06)	0.61 (-0.17,1.39)	0.58 (-6.26,7.42)	1.02 (0.30-1.74)**
NHCl ₂ water (µg/L)		-0.50 (-1.59, 0.57)	0.12 (-2.42,2.67)	-0.01 (-0.02,0.01)	0.92 (-0.03,1.91)	-0.10 (-0.71,0.51)	2.93 (-2.10,7.97)	-0.84 (-1.38,-0.33)**
CHCl ₃ exhaled breath (µg/m ³)		0.48 (-0.78, 1.74)	0.73 (-2.25,3.72)	0.00 (-0.02,0.02)	0.14 (-1.07,1.35)	0.59 (-0.17,1.32)	0.34 (-5.69,6.37)	0.24 (-0.44,0.92)
CHCl ₂ Br exhaled breath (µg/m ³)		0.14 (-0.77, 1.06)	-0.43 (-2.60,1.73)	0.00 (-0.01,0.02)	0.22 (-0.66,1.10)	0.43 (-0.13,0.99)	0.87 (-3.50,5.25)	0.55 (0.08,1.02)*
CHClBr ₂ exhaled breath (µg/m ³)		-0.05 (-1.79,1.69)	-1.07 (-5.18,3.03)	0.01 (-0.02,0.03)	0.22 (-1.43,1.88)	0.71 (-0.29,1.70)	1.06 (-7.22,9.35)	1.92 (1.19,2.67)**
CHBr ₃ exhaled breath (µg/m ³)		0.35 (-0.97,1.65)	-0.05 (-3.16,3.06)	0.00 (-0.02,0.02)	-0.07 (-1.53,1.38)	0.32 (-0.41,1.05)	3.13 (-3.09,9.30)	1.21 (0.59,1.82)**
TTHMs exhaled breath (µg/m ³)		0.31 (-0.79,1.44)	0.17 (-2.49,2.81)	0.00 (-0.02,0.02)	0.17 (-0.93,1.24)	0.54 (-0.12,1.20)	0.76 (-4.57,6.10)	0.59 (0.02,1.18)*
Energy expenditure (Kcal)		-0.65 (-2.03,0.73)	-2.97 (-6.10,0.17)	0.02 (-0.00,0.04)	0.20 (-1.14,1.54)	0.98 (0.22,1.74)*	0.85 (-5.73,7.41)	1.04 (0.37,1.73)**

IQR: interquartile range. % pred FEV-1: Percentage of forced expired volume in 1 second from the predicted by age, gender and height. FVC: Forced vital capacity. SP-D: Surfactant protein D. CC16: Clara cell secretory protein. SP-D and CC16 were measured in serum. FeNO: nitric oxide in exhaled breath. 8-isoprostane was measured in exhaled breath condensate. NHCl_2 : dichloramine; CHCl_3 : chloroform; CHCl_2Br : bromodichloromethane; CHClBr_2 : dibromochloromethane; CHBr_3 : bromoform; TTHMs: total trihalomethanes.

a. Wilcoxon test. b. Beta coefficients from linear regression models represent a change in the biomarker level for an increase from percentile 25 to percentile 75 of the exposure parameter. FeNO models are adjusted for rhinitis; 8-isoprostane models are adjusted for usual swimming pool attendance. The other models are crude.

*p-value <0.05 **p-value <0.01

Table 4. Concentration of 8 cytokines and VEGF (pg/mL) in exhaled breath condensate before and after swimming

	RANTES	Ip10	VEGF	TNF	IL12p70	IL-10	IL-8	IFN- γ	IL-4
N ^a	31	36	44	39	39	43	40	32	38
Before. Median (IQR)	0.85 (0.0,2.0)	1.18 (0.1,2.5)	3.63 (1.5,8.2)	0.89 (0.4,1.7)	0.66 (0.0,1.0)	0.89 (0.2,1.5)	1.24 (0.7,2.2)	1.29 (0.0,1.8)	0.70 (0.3,2.0)
After. Median (IQR)	1.17 (0.4,1.6)	1.33 (0.0,2.2)	4.41 (2.3,7.1)	0.71 (0.4,1.6)	0.31 (0.0,0.7)	0.84 (0.0,1.5)	1.42 (0.4,2.2)	1.25 (0.0,2.4)	0.72 (0.3,1.1)
Change. Median (IQR)	-0.20 (-0.8,1.1)	-0.08 (-1.3,1.3)	0.11 (-3.4,4.0)	-0.16 (-0.7,0.7)	0.00 (-0.6,0.0)	-0.15 (-0.6,0.7)	0.00 (-0.7,0.4)	-0.02 (-1.5,1.5)	-0.07 (-1.2,0.7)
p-value (change \neq 0) ^b	0.631	0.683	0.879	0.477	0.107	0.740	0.898	0.903	0.658

IQR: interquartile range.

a. Samples with undetectable levels were imputed half of the detection limit. Participants with undetectable levels before and after swimming were excluded. b. Wilcoxon test.

Table 5. Multiple linear regressions between serum CC16 concentration ($\mu\text{g/L}$) in relation to a unit increase in indicators of DBP exposure and energy expenditure

Model	Variables in the model	B (95%CI)	p-value	R ²	N
1	CHClBr ₂ exhaled breath ($\mu\text{g/m}^3$)	1.68 (0.93,2.43)	<0.001	0.45	46
	Energy expenditure (Kcal)	0.69 (0.09,1.28)	0.024		
2	CHClBr ₂ exhaled breath ($\mu\text{g/m}^3$)	1.49 (0.65,2.33)	0.001	0.46	46
	Energy expenditure (Kcal)	0.68 (0.08,1.27)	0.027		
	Free chlorine (mg/L)	0.33 (-0.35,1.01)	0.336		
3	CHCl ₂ Br exhaled breath ($\mu\text{g/m}^3$)	0.45 (0.01,0.89))	0.047	0.26	46
	Energy expenditure (Kcal)	0.97 (0.30,1.64)	0.005		
4	CHCl ₂ Br exhaled breath ($\mu\text{g/m}^3$)	0.35 (-0.09,0.78)	0.117	0.34	46
	Energy expenditure (Kcal)	0.87 (0.22,1.51)	0.010		
	Free chlorine (mg/L)	0.77 (0.08,1.46)	0.030		
5	CHBr ₃ exhaled breath ($\mu\text{g/m}^3$)	0.98 (0.31,1.65)	0.005	0.33	46
	Energy expenditure (Kcal)	0.66 (-0.03,1.35)	0.060		
6	CHBr ₃ exhaled breath ($\mu\text{g/m}^3$)	0.82 (0.16,1.48)	0.016	0.39	46
	Energy expenditure (Kcal)	0.61 (-0.05,1.28)	0.070		
	Free chlorine (mg/L)	0.69 (0.03,1.36)	0.042		
7	TTHMs exhaled breath ($\mu\text{g/m}^3$)	0.46 (-0.10,1.02)	0.105	0.24	46
	Energy expenditure (Kcal)	0.97 (0.29,1.65)	0.006		
8	TTHMs exhaled breath ($\mu\text{g/m}^3$)	0.30 (-0.25,0.86)	0.274	0.32	46
	Energy expenditure (Kcal)	0.88 (0.22,1.53)	0.010		
	Free chlorine (mg/L)	0.79 (0.08,1.49)	0.030		
9	Free chlorine (mg/L)	0.85 (0.16,1.54)	0.017	0.28	47
	Energy expenditure (Kcal)	0.90 (0.25,1.56)	0.008		

CHClBr₂: dibromochloromethane; CHCl₂Br: bromodichloromethane; CHBr₃: bromoform;

TTHMs: total trihalomethanes. No other variables were included in the models.

Figure legends

Figure 1. Study design and timing of the sample collection and in-situ measurements.

Legend:

* Urine was collected for genotoxicity analysis (Kogevinas et al. submitted).

Figure 2. Correlation between the change in serum CC16 concentration and CHClBr_2 in exhaled breath, energy expenditure during swimming, free chlorine in water and dichloramine in water.

Figure 3. Concentration of FeNO and serum CC16 before (B) and after (A) swimming, stratified by atopic status. Median and interquartile range.

Legend:

Phadiatop test was used to define atopic status.

p-value from a Mann-Whitney test between atopics and non-atopics: 0.022 for FeNO and 0.560 for CC16.

No swimming in pools (1 week)			
<i>Time (min)</i>			
0	<i>Pre-exposure</i>	THMs in exhaled breath	
15		Exhaled breath condensate	
40		Fractional exhaled NO	
50		Spirometry	
60		Peripheral blood extraction	
<i>Time (min)</i>			
85	Swimming 40 minutes		0
130	<i>Post-exposure</i>	THMs in exhaled breath	5
145		Quick shower + dressing	20
160		Exhaled breath condensate	35
185		Spirometry	60
195		Peripheral blood extraction	70
205		Fractional exhaled NO	80
215		Questionnaires	90

Figure 1.

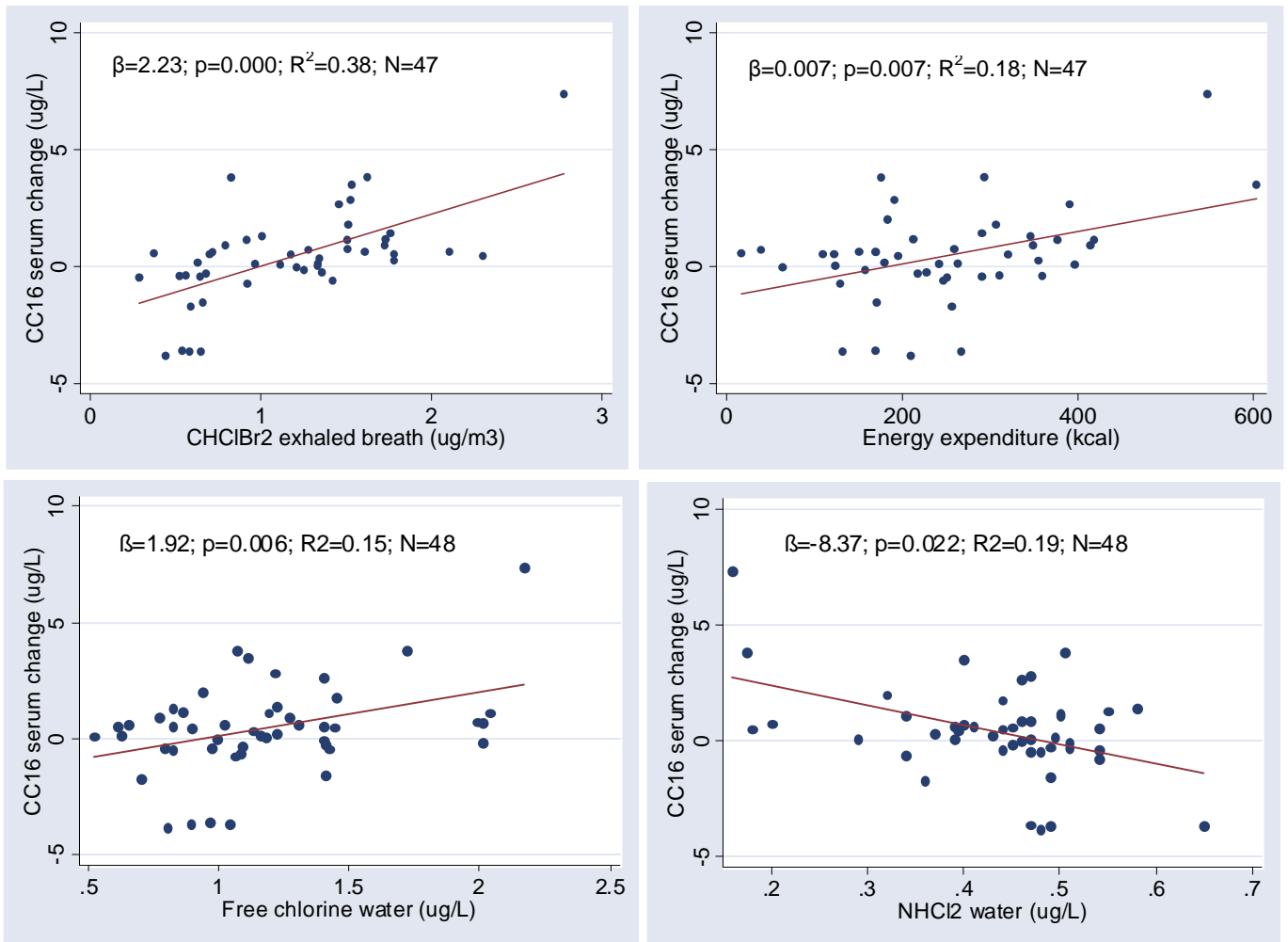


Figure 2.

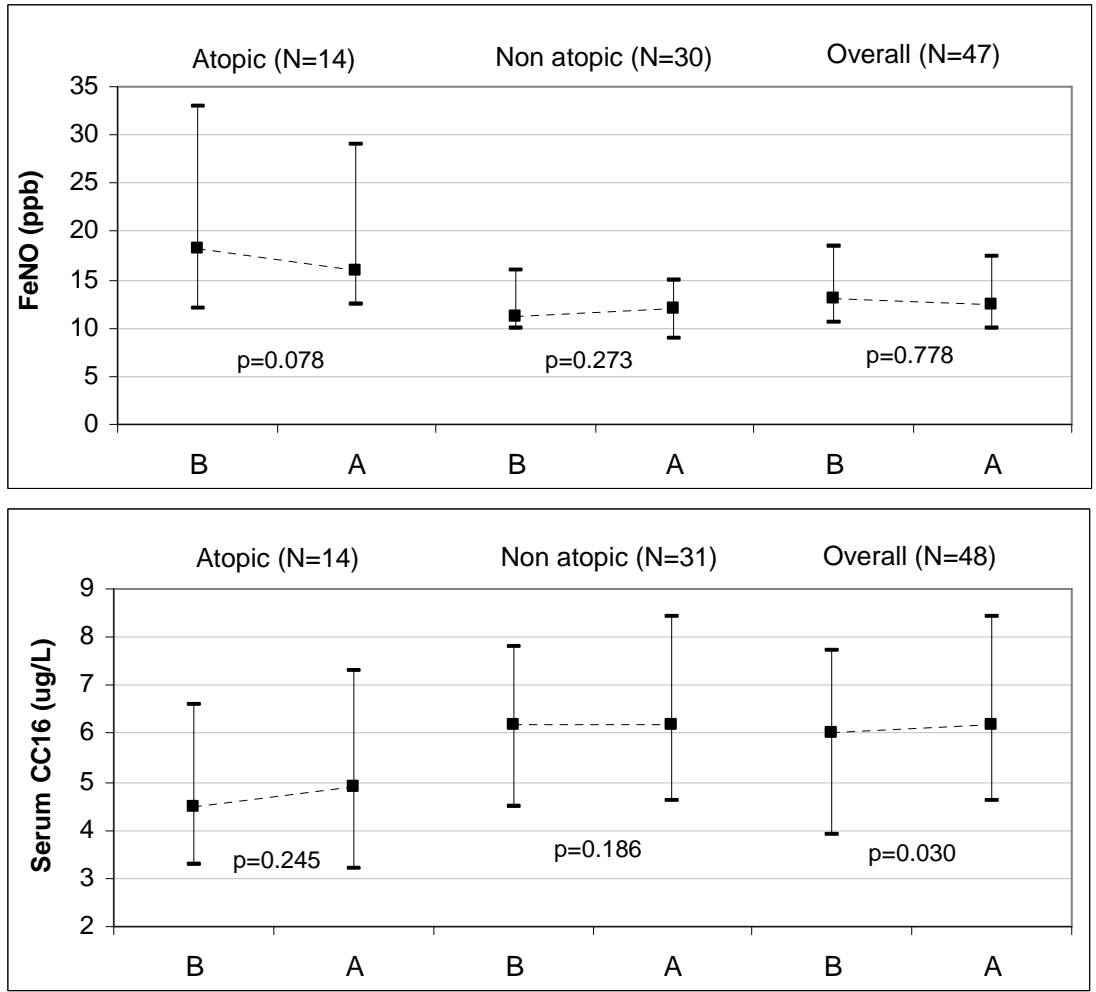


Figure 3.