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Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2012-000973
Article Type:	Research
Date Submitted by the Author:	31-Mar-2012
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Primary Subject Heading:	Respiratory medicine
Secondary Subject Heading:	Public health
Keywords:	PUBLIC HEALTH, RESPIRATORY MEDICINE (see Thoracic Medicine), OCCUPATIONAL & INDUSTRIAL MEDICINE

20120331

Lung function in volunteers before and after exposure to trichloramine in indoor pool environments and asthma in a cohort of pool workers

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List of abbreviations:

CC16: Clara Cell protein 16

FEV_1: Forced Expiratory Volume in 1 second, liters

$FEV_{\%}$: FEV_1x100/FVC

FVC: Forced Vital Capacity, liter

NCl_3: Nitrogen trichloride or trichloramine

OR: Odds Ratio

RHINE: Respiratory Health in Northern Europe

SPD: Surfactant protein D

Summary:

1) Article focus:

Exposure to trichloramine (NCl_3) in swimming-pool air is known to cause mucous membrane and pulmonary effects, but statistically significant changes in lung function among adults have not been reported.

Epidemiological studies of asthma among pool workers are not available.

2) Key messages:

In this study we found for the first time, statistically significant decreases in lung function in volunteers after exposure to pool air with commonly occurring levels of NCl_3.

We found a tendency towards a higher odds ratio (OR) for asthma in a nested case reference study within a cohort of 1102 pool workers.

Our findings support the notion that current workroom exposures of NCl_3 may contribute to asthma development.

3) Strengths and limitations: This is the first study showing small but statistically significant decreases in lung function after exposure to pool air. This is the first nested Case/Control study in pool workers. It reports an OR for asthma of 2.31 (95% CI 0.79-6.74) among pool workers with the highest exposure (after correction for heredity), but this finding did not reach statistical significance.

LUNG FUNCTION and ASTHMA in POOL ENVIRONMENTS

ABSTRACT

Objectives: Exposure to trichloramine (NCl_3) in indoor swimming pool environments is known to cause mucous membrane irritation, but if it gives rise to changes in lung function or asthma in adults is not known. 1: We determined lung function in volunteers before and after exposure to indoor pool environments 2: We studied the occurrence of respiratory symptoms and asthma in a cohort of pool workers.

Design/Methods/Participants: 1. We studied two groups of volunteers, 37 previously non-exposed healthy persons and 14 pool workers, who performed exercise for two hours in an indoor pool environment. NCl_3 in air was measured during pool exposures and in 10 other pool environments. Filtered air exposures were used as controls. Lung function and biomarkers of pulmonary epithelial integrity were measured before and after exposure. 2. We mailed a questionnaire to 1741 persons who indicated in the Swedish census 1990 that they worked at indoor swimming-pools.

Results: 1. In previously non-exposed volunteers, statistically significant decreases in FEV_1 and $FEV_{\%}$ (p=0.01 and p=0.05 respectively) were found after exposure to pool air (0.23 mg/m^3 of NCl_3). In pool workers, a statistically significant decrease in $FEV_{\%}$ (p=0.003) was seen after exposure to 0.15 mg/m^3 of NCl_3. In the 10 other pool environments the median NCl_3 concentration was 0.18 mg/m^3. 2. Our nested Case/Control study in pool workers found an OR for asthma of 2.31 (95% CI 0.79-6.74) among those with the highest exposure. Exposure-related acute mucous membrane and respiratory symptoms were also found..

Conclusions: This is the first study in adults showing statistically significant decreases in lung function after exposure to NCl_3. An increased OR for asthma among highly exposed pool workers did not reach statistical significance, but the combined evidence supports the notion that current workroom exposures may contribute to asthma development. Further research on sensitive groups is warranted.

INTRODUCTION and OBJECTIVES

Mono-, di- and trichloramines are formed following a reaction between ammonia (NH_3) or other nitrogen containing substances present in swimming pool water when hypochlorite is used as a disinfectant. Trichloramine (NCl_3) is the most volatile chloramine and is emitted into the air of indoor swimming pools. Exposure to this substance was the suspected cause of outbreaks of short-incubation ocular and respiratory illness [1,2], but concentrations of NCl_3 in pool environments were not known in these outbreaks. It is known, however, that acute respiratory and eye symptoms may occur among recreational swimmers in relation to measured levels of NCl_3 in pool environments [3].

Only few and inconclusive studies have been performed on lung function among adults after exposures to measured levels of NCl_3 in pool environments [4,5] and additional studies are required.

Clara cell protein 16 (CC16) is an epithelial protective protein in peripheral lung tissue and changes in its serum levels are used as a biomarker of epithelial integrity [6]. It has been shown to be decreased in relation to frequency of pool attendance [7]. However, changes in serum levels of CC16 have not been studied after short term exposure to NCl_3.

Thickett et al 2002 [8] reported three cases of occupational asthma among British pool workers exposed to NCl_3. There is a lack of epidemiological studies on asthma among those working in swimming pool environments.

The objectives of the present study were 1. To perform a controlled human exposure study of lung function and biomarkers of pulmonary epithelial integrity in volunteers before and after

exposure to indoor swimming pool environments. 2. To perform an epidemiological study of self-reported asthma and subjective symptoms in a cohort of indoor swimming pool workers

DESIGN, MATERIALS AND METHODS

Air sampling and determination of NCl_3:

Exposure measurements in human exposure study

In the two pool environments where our study of volunteers and pool-workers took place hypochlorite was used as disinfectant. Air samples were collected in the breathing zone: one sample for each 2-hour exposure, in total 51 samples.

Determination of NCl_3 at other indoor swimming pools:

Additional determinations of NCl_3 were performed 2004-2008 at 10 different pool establishments (7 conventional ones and 3 "adventure water lands") in northern Sweden with totally 30 indoor pools. Hypochlorite was used as disinfectant. At each swimming-pool, air was sampled during 3 hours at 3 to 4 different locations in close vicinity of the pool. The equipment was mounted on a stand with the filter at a height of approximately 1.5 meter. Sampling was performed on three different days during winter and three different days during summer.

Air collection and Analysis: 1L/min of air was pumped through a filter (quartz filter QM-A 37 mm Whatman International Ltd., Maidstone, England). The filter was soaked in a solution of sodium carbonate and arsenic trioxide (AsO_3) and dried as presented earlier [9]. When NCl_3 is collected on the filter it is reduced to chloride ion (Cl^-) [9]. After sampling, the filters

were extracted with 10 ml of ultra-pure water, shaken for 30 minutes and filtered through a 13 mm syringe filter (IC Acrodisc®, PALL). The chlorides were analyzed in a suppressed ion chromatography system (Triatlon 900 autosampler, Spark, The Netherlands); ICSep AN1, Anion column (CETAC, Omaha, USA); SCX membrane suppressor column (Sequant, Umeå, Sweden); JD-21 conductivity detector (Costech Microanalytical Ltd, Tallin, Estonia)). The eluent was 7.5 mM NaOH and the suppressor 5 mM H_2SO_4. Control samples of two known chloride concentrations (0.5, 3.0 $mg \cdot l^{-1}$) and at least two blanks were run together with the samples in each run. The chloride concentrations in the blanks were subtracted from the concentration in the samples. The detection limits of NCl_3 (1.78 and 1.18 $\mu g \cdot m^{-3}$ for 2 h and 3 h samplings, respectively) were determined as three times the mean standard deviation of the amount collected on filters of 10 blanks. The limits of quantification (5.9 $\mu g \cdot m^{-3}$ and 3.9 $\mu g \cdot m^{-3}$ for 2 h and 3 h samplings respectively) were determined as ten times the mean standard deviation for the same blanks.

Human exposure study

Study groups:

Group A: 37 healthy subjects (20 men and 17 women, mean age 24.5 years). They were not regular swimming pool visitors and they had not visited a swimming pool within four weeks before study start.

Group B: 14 workers at swimming pools (5 men, 9 women, mean age 39.9 years).

All participants were non-smokers with normal lung function and had no history of allergy or pre-existing lung disease. Subjects were free of airway infection for ≥4 weeks prior to the first exposure and throughout the remainder of the study.

Study design

The study was conducted in a crossover control fashion. Each volunteer was exposed to filtered air in an exposure chamber and on another occasion to an indoor pool environment. In the exposure chamber, located in a separate building away from swimming-pools, incoming air was adjusted to room temperature and filtered through a particle filter. The exposures were performed in random order. Successive exposures were separated by ≥2weeks. The exposures (pool environment or filtered air) lasted for 2 hours. The study subject was exercising on a bicycle ergometer with moderate exercise (minute ventilation 20 $L \cdot min^{-1} \cdot m^{-2}$), during 15-minutes followed by 15 minutes of rest, i.e. four periods of exercise and four periods of rest.

Lung function:

FVC and forced expiratory volume in 1 sec (FEV_1) was determined using a portable spirometer connected to a computer (KoKo Spirometer and KoKo DigiDoser; Pulmonary Data Service Instrumentation, Inc, Louisville, KY, USA), calibrated in the morning and after every 10^{th} measurement. $FEV_{\%}$ was calculated as a percentage of FVC ($FEV_{\%}=FEV_1 x100/FVC$). Lung function was measured immediately before and after exposure in a room with non-detectable levels of NCl_3 (< 0.002 mg NCl_3/m^3) or in a room adjacent to the exposure chamber.

Blood sampling and determination of biomarkers.

We obtained blood samples from the antecubital vein at 0 h and 2 h, i.e. before and after exposure, and at 4, 6 and 8 hours. Peripheral blood was collected into BD Vacutainer tubes (BD, Plymouth, UK). Each sample was allowed to clot for 1-2 h at room temperature, centrifuged at 3,000x*g* and serum was transferred to cryotubes and frozen at –80°C. These samples were sent to the Industrial Toxicology Unit at the Catholic University of Louvain in Brussels (IUTUCL), Belgium for determination of Clara Cell protein 16 (CC16) and

Surfactant Protein D (SPD). CC16 was determined by latex immunoassay using a rabbit anti-CC16 antibody (Dakopatts, Glostrup, Denmark) and CC16 purified at (IUTUCL) as standards [10,11]. All samples were run in duplicate at two different dilutions. The between- and within-run coefficients of variation range 5–10% and results are comparable with ELISA methods [12]. SPD determinations were performed using the Biovendor ELISA kit (Biovendor, Heidelberg, Germany). Analyses were done in duplicate as recommended by the manufacturer.

Total IgE was determined in human serum by a double antibody sandwich ELISA method (Human IgE ELISA kit, Immunology Consultants Lab; Inc, Newberg, OR). The quantity of IgE in the samples was interpolated from a standard curve.

Statistical analyses

All data from CC16 measurements were corrected for diurnal variation according to Helleday et al 2006 [13] and recalculated to correspond to 7 AM. $CC16(corr) = CC16 + 0.582*T - 0.032*T^2$. T is the time after 7.00 AM when the blood sample was taken. Because CC16 values are highest in the morning [13], corrected CC16 values were somewhat greater than measured values.

Statistics: We used repeated measures analyses of variance (Huynh-Feldt corrected) with time and exposure as within-subject factors and group as between-subject factor. Paired t-test or Wilcoxon signed rank test was used when comparing exposures to filtered air and pool environment at baseline (0 hrs) and after exercise (2h). Median IgE values were compared by the Westenberg-Mood median test. SPSS version 17.0 was used to perform the statistical analyses. A p-value of 0.05 was considered statistically significant.

Epidemiological study

Population:

The epidemiological study group included 1741 persons in the Swedish Census of Population and Housing 1990 who had indicated that they worked at swimming pools. Early 2007 a questionnaire was mailed to them. There was one reminder.

Questionnaire: Questions dealt with time periods in various jobs, time spent in swimming pool environments, various symptoms from the respiratory tract and mucous membranes of the eyes and possible use of medication for asthma. 589 women and 513 men, age 30 ->80 years responded. Among 50 non-responders, interviews were performed via telephone. There was a lower prevalence of asthma and respiratory symptoms among the non-responders, not statistically significant.

In a nested case-control study within this cohort, 44 cases of self reported asthma occurred after the person was hired as a pool worker. 128 age and sex matched controls were selected within the cohort.

Exposure assessment:

Based on information on work titles given by each individual, exposure was classified into three different categories; 0, 1, or 2. 0 stands for no exposure, 1 for low exposure and 2 for high exposure. The exposure level is not an estimate of the concentration of NCl_3 in air but is based on the average time during a workday the individual spent in the pool area. Those within category 0 did not spend any time in a pool area, e.g. a cashier. A person within category 1 did occasionally spend some time in the pool area. A manager of a swimming pool or a technician belongs to this category. Individuals belonging to category 2 were those

spending most of the workday in the pool area, e.g. a swimming teacher, or a swimming pool worker.

Comparison data

We obtained data on asthma in 1990 from the study "Respiratory Health in Northern Europe" (RHINE [14]) via one of the authors of the present paper (BF). As we used the same questions in the present study as in RHINE, it was possible to derive adequate sex and age stratified comparison data up to the age of 55.

Statistics

Fisher´s test was used to test differences between proportions. Conditional logistic regression was used for analyses in the nested case-control study and logistic regression for analyses of asthma in relation to years worked in swimming-pool environments. All statistical analyses were performed using the statistical package R, version 2.9.0 (www.r-project.org). P-values equal or less that 0.05 were considered statistically significant.

Ethics

The project was approved by the Regional ethical review board in Umea, Sweden (Dnr 05-044M) and volunteers provided written informed consent. The study was carried out according to the declaration of Helsinki.

RESULTS

Air sampling.

Experimental exposure (Human exposure study)

The NCl_3 levels during the experimental exposures were

Group A: Mean 0.23 mg/m^3 (SD 0.09)

Group B Mean 0.15 mg/m^3 (SD 0.04)

Other Swimming pools

NCl_3 concentrations in air at the 10 different indoor swimming pool establishments were between 0.001- 0.77 mg/m^3, median 0.18 mg/m^3, arithmetic mean (AM) 0.21 mg/m^3 (n=129). The AM concentrations of NCl_3 in each of the ten different pool establishments were between 0.09 – 0.32 mg/m^3. There was no difference in NCl_3 concentrations during summer compared with winter conditions (results not shown).

Human exposure study

Lung function

Group A:

Measured FEV_1 volumes among healthy volunteers as well as the difference before and after 2 hours of exposure to pool environment or filtered air are summarized in Table 1. There was a small, statistically significant decrease (p=0.01) in FEV_1 (mean decrease = 0.05 L) after exposure to swimming pool air. After exposure to filtered air there was a slight, not statistically significant increase in FEV_1 (mean increase 0.01 L). When comparing the differences (Δ-values) in FEV_1 before and after exposure to pool environment with the Δ-values for exposure to filtered air in the same individuals, the difference between Δ-values was statistically significant (p=0.01).

$FEV_{\%}$ values among healthy volunteers are also given in table 1. After exposure to pool air, there was a small decrease (0.8 $FEV_{\%}$) that was marginally statistically significant (p=0.05).

After exposure to filtered air, there was a small (statistically non-significant) increase in $FEV_{\%}$ values. When the individual differences (Δ-values) of $FEV_{\%}$ before and after exposure to pool air were compared with the corresponding Δ-values in filtered air, a statistically significant difference was demonstrated (p=0.004, paired t-test). Airway obstruction is usually defined as $FEV_{\%}$ below 70 (www.goldcopd.com). Only one value was below 70 (after exposure) among the healthy volunteers.

Group B

In table 2, FEV_1 values for the swimming-pool workers are summarized. After exposure to pool air there was a small and not statistically significant decrease in FEV_1, 0.01 L. There was also a small decrease in FEV_1 after exposure to filtered air (0.05 L, p=0.054). When considering the $FEV_{\%}$ values for the workers (Table 2) before and after exposure to pool air, there was a statistically significant decrease of 1.36% (p=0.003). After exposure to filtered air the small decrease in $FEV_{\%}$ of 0.43% was not statistically significant. Only two FEV% values among the pool workers (one before and one after exposure) were below 70.

Biomarkers of pulmonary epithelial integrity:

Group A

Mean CC16corr values and related standard deviations (SD) in previously unexposed healthy volunteers, are shown in Figure 1 for 33 of the participants in group A. For the remaining 4 persons, values were missing and they were therefore excluded from analysis.

At baseline (0 hrs), mean CC16corr = 12.6 µg/L before pool exp (0 h) and 10.3 µg/L immediately before (0 h) exposure to filtered air. This difference (p=0.018, paired t-test) is difficult to explain because the same volunteers were exposed to both pool environment and filtered air and they were randomly assigned to either exposure.

Group B

Results are shown in Table 1. The mean CC16corr was 6.5 µg/L before both pool and filtered air exposures.

The difference between groups A and B persisted during and after exposure (0-8 hrs) and is statistically significant ($p<0.001$ repeated measures analysis of variance on log transformed data). There is also a different change with time. Group A decreases with time and group B increases with time. The difference in trend is statistically significant $p=0.038$.

The decrease with time in group A during and after exposure to pool environment as well as filtered air is statistically significant ($p<0.05$, GLM repeated analysis model). There is no statistically significant difference in change with time between pool exposure and filtered air. For improved analysis, values were converted to their natural logarithms, SDs decreased, providing improved statistical conditions, but no statistically significant effect of exposure could be shown (data not shown).

SPD values, shown in Figure 2, also display a change with time, with lower values with increasing time intervals from initiation of exposure.Considering the log transformed SPD variable, there was a difference ($p<0.05$) before and after exposure (i.e. SPD values were higher at 0 hrs than at 2 hrs) and there was a further decrease ($p<0.01$) with time at 2 hrs – 8 hrs (Figure 2). This decrease was similar for exposure to pool air and filtered air. We found no statistically significant changes in SPD values in relation to exposure.

IgE

The median IgE value was 1.0 mg/L in Group A and 0.0 in group B, compared to 3.0 mg/L in mild asthmatics (n=18) participating in another study on influence of general air pollution conducted by one of the authors (BF). Compared to the volunteers in the present study (groups A and B), the median value of the asthmatics was statistically significantly higher (p= 0.002) based on Westenberg-Mood median test.

Epidemiological study

There was a statistically significant relationship between the number of hours, during an average day, spent in the swimming pool environment and the incidence of acute symptoms ($p<0.01$; logistic regression). Frequent symptoms were: dyspnoea (13%), cough (23%), nose irritation (29%), throat irritation (24%) and eye irritation (37%).

The prevalence of self reported asthma attacks or medication for asthma was higher ($p<0.01$; Fisher's test) among swimming pool workers in this study (12.3%) compared with the reference group 8,1% (RHINE 1999). When considering rates (age and sex adjusted) by logistic regression, there was still a higher prevalence among swimming-pool workers, but less significant (p=0.11).

In the nested case-control study, the Odds Ratio (OR) for asthma was 2.53 (95% CI 0.89 – 7.19) for persons with exposure level 2 compared with persons exposed to level 0 or 1. After correction for heredity, the corresponding numbers were: OR 2.31 (95%CI 0.79-6.74). These values refer to cases of self reported asthma occurring after they started pool work, compared with controls.

A tendency to a reduced risk of developing asthma in relation to the number of years of work in swimming-pool environments was indicated among individuals who worked more than one year and developed asthma after they started to work in such environments. This tendency was, however not, statistically significant p=0.07.

Discussion

Our observations of statistically significant decreases in FEV_1 and $FEV_{\%}$ in previously non-exposed volunteers and pool workers after exposure to pool air are the first such observations in adults. Carbonelle et al 2002 [4] reported an increase in FEV_1/VC among children and a non-statistically significant decrease in adults (n=13) after they had attended a chlorinated pool. Carbonelle et al 2008 [4] found FEV_1/VC to be unchanged in 11 young adults after swimming in a non-chlorinated pool and slightly, but not statistically significantly decreased after swimming in a chlorinated pool. The lack of statistically significant decrease may be related to the fact that only 11 adults were studied[4], while the statistically significant decrease in our study was based on 37 previously unexposed healthy volunteers. Very few FEV% values were below 70 (indicating no clinically significant airway obstruction within the study group). The reduction in FEV% seen after exposure in pool air here, albeit small, may be a sign of an obstructive airway effect. In children, Bernard et al 2003 [14] found a statistically highly significant relationship between cumulative pool attendance during kindergarten and PEF 15 (post exercise reduction of peak expiratory flow by 15 percent), providing supportive evidence of airway effects of exposure to chlorinated pool environments. .

CC16 levels in serum increase when lung epithelium permeability is adversely affected by air pollutants or other lung toxicants [6, 10,15, 16]. On the other hand, reduced levels of CC16 in

lung lavage fluid occur in several lung disorders, probably due to a decrease in the production of CC16 as a consequence of a depletion of Clara cells [17]. We found a statistically significant difference in the serum level of CC16 between pool workers compared to volunteers. This finding is consistent with our previous finding of a lower CC16 value in school children frequently attending indoor swimming pools than in those with a low attendance at such pools [5]. The difference between workers and previously unexposed healthy volunteers like the difference among school children may be due to a depletion of Clara cells. We did not find any statistically significant exposure-related changes in concentrations of the biomarkers of pulmonary epithelial integrity (CC16 and SPD) after exposure to pool air for 2 hours. The lack of such an exposure-related change was probably due to the relatively short exposure duration and low exposure level of NCl_3. Another possible explanation is that NCl_3 acts preferentially in the more proximal parts of the respiratory tract, inducing a mild constriction of the central airways, but with less interference in the terminal bronchioles, where the Clara cells are located. In previous studies of volunteers exposed to ozone [6], we found both a decrease in FEV_1 and an increase in serum CC16 concentrations after exposure.

All CC16 values in the present study were corrected for diurnal variation [12]. In spite of such correction, there was a statistically significant decrease with time of experiment from 0 h to 8 h in group A (regardless of exposure to NCl_3). This indicates that the real diurnal variation exceeded the one assumed in the employed correction calculation. For group B there is an opposite trend with time, possibly related to an inadequate correction of the values in this group. The pool workers were older and had been more exposed to NCl_3 during many years of work in pool environments. Data on diurnal variation for SPD are not available in the

literature. Our data, with a statistically significant decrease with time between 0 h and 8 h, indicate that a diurnal variation exists.

The absence of exposure-related effects (after 2 hours exposure) on serum concentrations of CC16 and SPD in combination with small, statistically significant decreases in FEV_1 and $FEV_{\%}$ show that the 2-hour exposure level in this experiment can be regarded as the Lowest-Observed-Adverse-Effect-Level on the lung for this group of volunteers. It should be borne in mind that individuals with increased sensitivity to adverse respiratory effects, like those with pre-existing asthma, were not included in the present study. Our observation may be of use in relation to administrative action in setting exposure limits for NCl_3. To our knowledge, no health-based limit values for occupational or environmental exposures have yet been set for NCl_3. A technical value of 0.2 mg/m^3 was recently recommended in Germany [18]. Bernard et al 2006 [19] showed that serum total IgE was a factor determining the risk of adverse pulmonary effects after exposure to pool environments. Serum levels of total IgE in the volunteers and workers of our study were lower than among mild asthmatics. The absence of an increased level of total serum IgE among the present volunteers indicates that individuals with possibly increased sensitivity due to increased IgE had been successfully excluded. Further studies on persons with elevated serum IgE would be of interest. Another group that may suffer respiratory effects at lower air concentrations of NCl_3 is competitive swimmers because their breathing volumes exceed those of the volunteers in the present study. Helenius et al 1998 [20] found increased respiratory symptoms and bronchial responsiveness in elite swimmers.

Our study indicates that employees in Swedish indoor pools are exposed to approximately the same level of NCl_3 as employees in France and Belgium. We found median NCl_3 concentrations of 0.18 mg/m^3 in ten different premises, while Hery et al 1995 [9] reported

0.14-0.91 mg/m^3 and Massin et al [3] reported a mean of 0.24 mg/m^3 in Public pool environments and 0.67 mg/m^3 in establishments with private owners. There are no previous published data on NCl_3 exposure in Swedish indoor pools. The work environment, i.e. ventilation and the use of sodium hypochlorite as disinfectant has probably not changed during the past decades. This makes it reasonable to estimate that pool workers have been exposed to NCl_3 at approximately the same levels as reported in this study.

In the epidemiological part of the present study, we found a statistically significant relationship between the number of hours spent in swimming pool environments and the incidence of symptoms. The workers reported a high incidence of respiratory and mucous irritation symptoms from 13 percent for dyspnoea to 37 percent for eye irritation. These findings are in accordance with previous observations in France [3] and Holland [1].

This study also found a higher prevalence of self-reported asthma in swimming pool workers than in a reference group. This difference remained when adjusted for age and sex, but failed to reach statistical significance ($p = 0.11$). Our nested case-referent study found an Odds Ratio (OR) for asthma of 2.53 (95% CI 0.89 – 7.19) for workers with more extensive exposure in pool areas (exposure level 2 compared to persons with exposure level 0 or 1). After correction for heredity the corresponding numbers were: OR 2.31 (95% CI 0.79 - 6.74). These values refer to cases of self-reported asthma occurring after they started to work in swimming-pool environments, compared to controls without asthma.

Cases of asthma in pool workers have been reported in the United Kingdom [8], but no epidemiological evidence has been reported. The findings of the present study did not reach statistical significance and provide only limited support for a causal relationship between asthma and work at indoor swimming pools. However, the fact that there was a tendency

towards a decreasing risk of asthma in workers with longer work history may indicate a healthy worker effect due to the irritating properties of NCl_3 in pool environments. A recent study [21] reported a higher prevalence (4.5%) of new-onset asthma among recreational swimmers with >320 hours of cumulative pool attendance compared to 0.4% among swimmers with <320 hours of pool attendance, thus supporting a role for exposure at chlorinated pools for development of asthma. In children engaged in recreational swimming, a statistically significant relationship was shown between cumulative attendance at indoor swimming pools and the probability of developing asthma in those with increased total IgE in serum [14,19]. Attendance at chlorinated pools before the age of 2 years increased the risk of bronchiolitis and asthma [22]

The present findings support the previously advanced hypothesis [7, 14, 19,21] that exposures to NCl_3 levels commonly occurring in indoor swimming pool environments can cause acute airway and mucosal symptoms as well as changes in lung function and deterioration of asthma.

Conclusions: For the first time in adults, statistically significant decreases in lung function were found in both previously unexposed subjects and pool-workers after exposure to pool air containing 0.23 and 0.14 mg/m^3 respectively, of NCl_3 compared to filtered air. The changes in lung function occurred in adults without any signs of allergy and with low IgE values. In a cohort of pool workers we found exposure-related acute mucous membrane and respiratory symptoms. An increased odds ratio for asthma (OR 2.31, 95% CI 0.79-6.74) was indicated in workers in the highest exposure category compared to lower exposures. Our observations give support to a previously advanced hypothesis that current exposures to NCl_3 can cause adverse

effects on mucous membranes and lungs of humans and contribute to the development of asthma. Further research in sensitive groups is warranted.

Data sharing: There is no additional data available

Acknowledgements: This work was supported by the Swedish Council for Working Life and Social Research (FAS) project 2004-0497 and the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS) project 2005-1561. The authors declare no competing interests.

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Figures and Tables

Table 1. Healthy volunteers (n=37): FEV_1 (forced expiratory volume, liter during 1 sec) and FEV% (FEV_1x100/forced vital capacity) measured before and after 2h exercise in filtered and pool air respectively. Mean ± SD. Mean differences (before-after) within parentheses.

Expiratory volume	**Exposure in filtered air**			**Exposure in pool air**		
	before	**after**	**mean diff Δ -values**	**before**	**after**	**mean diff Δ -values**
FEV_1	4.10 ± 0.85	4.11 ± 0.87	(-0.01)°	4.14 ± 0.87	4.09 ± 0.86	(0.05)**
FEV%	80.5 ± 5.8	80.9 ± 5.2	(-0.4)°	80.7 ± 5.3	79.9 ± 5.3	(0.8)*

**FEV_1 significantly lower after exposure to pool air, p = 0.01
*FEV% lower after exposure to pool air, p = 0.05
°difference not statistically significant

The FEV_1 Δ-values were -0.01 liter/sec in filtered air and 0.05 liter/sec in pool air, difference statistically significant, p = 0.01 (paired t-test).
Paired t-test of the difference in FEV% Δ-value in filtered air (mean -0.4 %) as compared pool s air (mean 0.8 %) was statistically significant, p = 0.004.

Table 2. Swimming pool workers (n=14): FEV_1 (forced expiratory volume, liter during 1 sec) and $FEV_{\%}$ (FEV_1x100/forced vital capacity) measured before and after 2h exercise in filtered air and pool air respectively. Mean ± SD. Mean differences (before-after) within parentheses.

Expiratory volume	Exposure in filtered air before	after	mean diff Δ -values	Exposure in pool air before	after	mean diff Δ -values
FEV_1	3.56 ± 0.99	3.51 ± 0.91	(0.05)°	3.59 ± 0.93	3.57 ± 0.92	(0.014) °
FEV%	78.86 ±6.3	78.43 ± 5.42	(0.43)°	79.1 ± 4.1	77.8 ± 5.1	(1.36)*

*FEV% lower after exposure to pool air, p = 0.003 (Wilcoxon signed rank test).
° indicates no statistically significant difference

Figure legends

Figure 1:Mean values (µg/L) and SD for CC16corr at various time points before (0h), immediately after exposure (2h) and the following 2 (4h), 4 (6h) and 6 hours (8h). Values are shown for the previously unexposed group of healthy volunteers (A) after exposure in a pool environment, after exposure to filtered air (two upper set of lines and bars). The two lower lines and related bars represent exposure in pool environment and filtered air for Group B, recruited among pool workers with several years exposure to pool environments**.**

Figure 2: Mean and SD for measured SPD values (µg/L) at various time points (0-8 hours) of the study. Exposure to pool environment or filtered air took place for 2 hours (between 0h and 2h). Group A: previously unexposed healthy volunteers. Group B: pool workers

Fig 1 (separate file)

Fig 2(separate file)

Statement:

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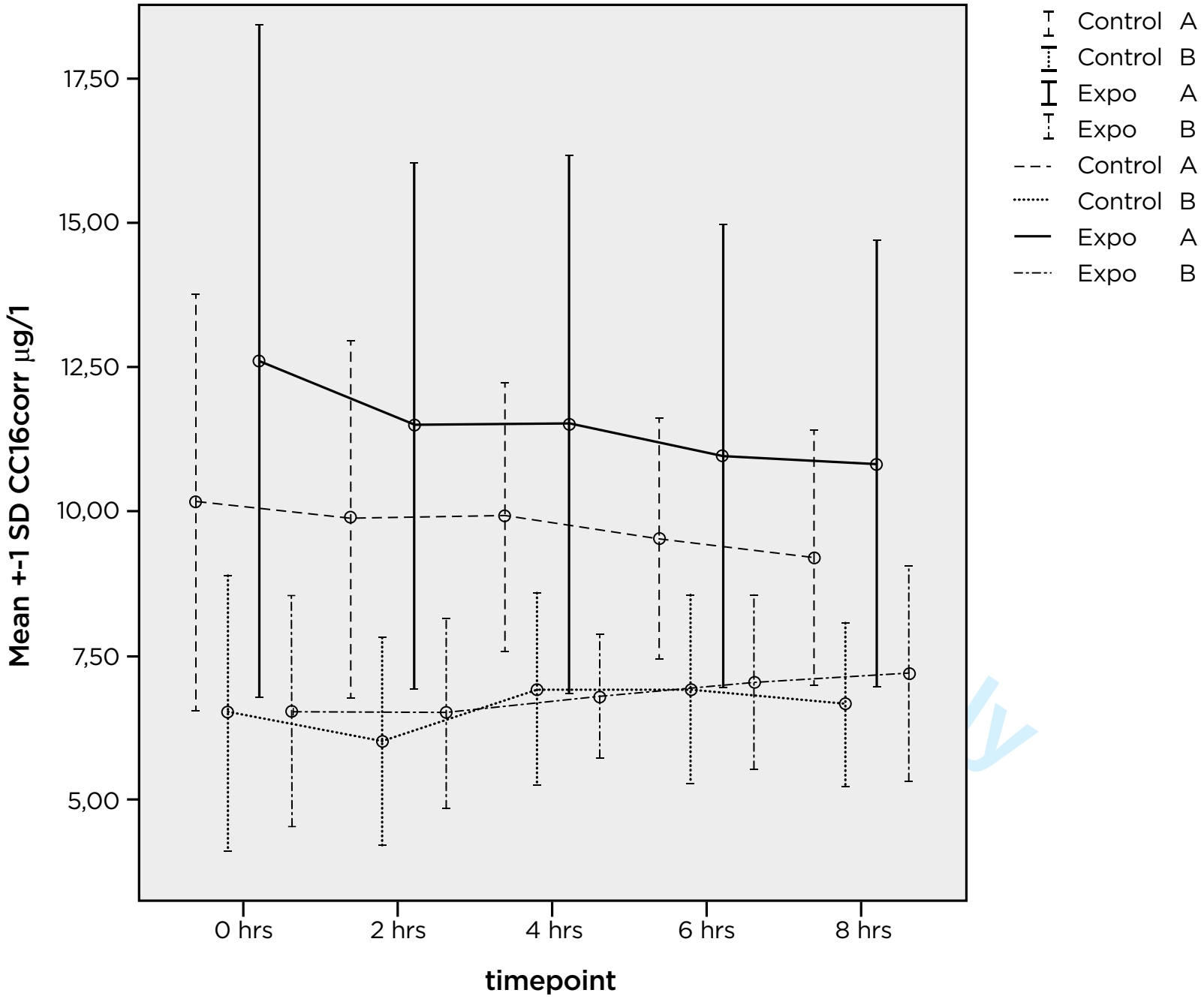
"The contributions of the authors are as follows: G.Nordberg 1,2,3; N-G.Lundstrom 1,2,3; B.Forsberg1,2,3; A.Hagenbjork-Gustafsson1,2,3; B. J-son Lagerkvist1,2,3; J. Nilsson1,2,3: M.Svensson1,2,3; A.Blomberg1, 2,3; L. Nilsson1,2,3; A. Bernard1,2,3; X. Dumont1,2,3; H.Bertilsson1,2,3 and K. Eriksson1,2,3.

1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published."


Mean +-1 SD CC16corr µg/1
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2 hrs
4 hrs
6 hrs
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timepoint
Control A
Control B
Expo A
Expo B
Control A
Control B
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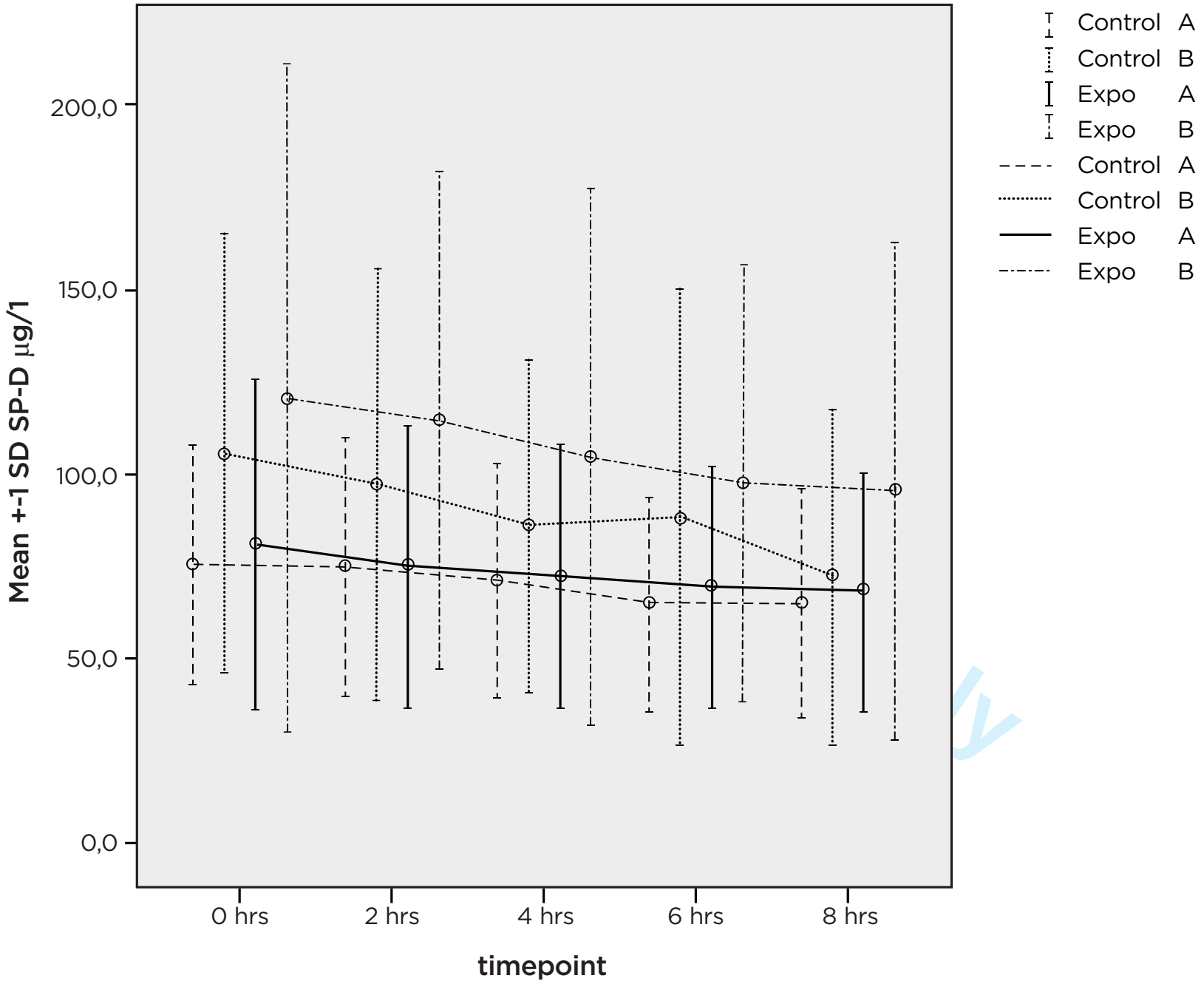
Control A
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0 hrs
2 hrs
4 hrs
6 hrs
8 hrs
timepoint

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported
Objectives	3	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	Present key elements of study design early in the paper
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses

Continued on next page

Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time
		Case-control study—Report numbers in each exposure category, or summary measures of exposure
		Cross-sectional study—Report numbers of outcome events or summary measures
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	18	Summarise key results with reference to study objectives
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ open

Lung function in volunteers before and after exposure to trichloramine in indoor pool environments and asthma in a cohort of pool workers

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2012-000973.R1
Article Type:	Research
Date Submitted by the Author:	02-Aug-2012
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Primary Subject Heading:	Respiratory medicine
Secondary Subject Heading:	Public health, Occupational and environmental medicine
Keywords:	PUBLIC HEALTH, RESPIRATORY MEDICINE (see Thoracic Medicine), OCCUPATIONAL & INDUSTRIAL MEDICINE

20120802

Lung function in volunteers before and after exposure to trichloramine in indoor pool environments and asthma in a cohort of pool workers

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List of abbreviations:

CC16: Clara Cell protein 16

FEV_1: Forced Expiratory Volume in 1 second, liters

$FEV_{\%}$: FEV_1x100/FVC

FVC: Forced Vital Capacity, liter

NCl_3: Nitrogen trichloride or trichloramine

OR: Odds Ratio

RHINE: Respiratory Health in Northern Europe

SPD: Surfactant protein D

Summary:

1) Article focus:

Exposure to trichloramine (NCl_3) in swimming-pool air is known to cause mucous membrane and pulmonary effects, but statistically significant changes in lung function among adults have not been reported.

Epidemiological studies of asthma among pool workers are not available.

2) Key messages:

In this study we found for the first time, statistically significant decreases in lung function in volunteers after exposure to pool air with commonly occurring levels of NCl_3.

We found a tendency towards a higher odds ratio (OR) for asthma in a nested case reference study within a cohort of 1102 pool workers.

Our findings support the notion that current workroom exposures of NCl_3 may contribute to asthma development.

3) Strengths and limitations: This is the first study showing small but statistically significant decreases in lung function after exposure to pool air. This is the first nested Case/Control study in pool workers. It reports an OR for asthma of 2.31 (95% CI 0.79-6.74) among pool workers with the highest exposure (after correction for heredity), but this finding did not reach statistical significance.

LUNG FUNCTION and ASTHMA in POOL ENVIRONMENTS

ABSTRACT

Objectives: Exposure to trichloramine (NCl_3) in indoor swimming pool environments is known to cause mucous membrane irritation, but if it gives rise to changes in lung function or asthma in adults is not known. 1: We determined lung function in volunteers before and after exposure to indoor pool environments 2: We studied the occurrence of respiratory symptoms and asthma in a cohort of pool workers.

Design/Methods/Participants: 1. We studied two groups of volunteers, 37 previously non-exposed healthy persons and 14 pool workers, who performed exercise for two hours in an indoor pool environment. NCl_3 in air was measured during pool exposures and in 10 other pool environments. Filtered air exposures were used as controls. Lung function and biomarkers of pulmonary epithelial integrity were measured before and after exposure. 2. We mailed a questionnaire to 1741 persons who indicated in the Swedish census 1990 that they worked at indoor swimming-pools.

Results: 1. In previously non-exposed volunteers, statistically significant decreases in FEV_1 and $FEV_{\%}$ (p=0.01 and p=0.05 respectively) were found after exposure to pool air (0.23 mg/m^3 of NCl_3). In pool workers, a statistically significant decrease in $FEV_{\%}$ (p=0.003) was seen (but no significant change of FEV_1) . In the 10 other pool environments the median NCl_3 concentration was 0.18 mg/m^3. 2. Our nested Case/Control study in pool workers found an OR for asthma of 2.31 (95% CI 0.79-6.74) among those with the highest exposure. Exposure-related acute mucous membrane and respiratory symptoms were also found.

Conclusions: This is the first study in adults showing statistically significant decreases in lung function after exposure to NCl_3. An increased OR for asthma among highly exposed pool workers did not reach statistical significance, but the combined evidence supports the notion that current workroom exposures may contribute to asthma development. Further research on sensitive groups is warranted.

INTRODUCTION and OBJECTIVES

Mono-, di- and trichloramines are formed following a reaction between ammonia (NH_3) or other nitrogen containing substances present in swimming pool water when hypochlorite is used as a disinfectant. Trichloramine (NCl_3) is the most volatile chloramine and is emitted into the air of indoor swimming pools. Exposure to this substance was the suspected cause of outbreaks of short-incubation ocular and respiratory illness [1,2], but concentrations of NCl_3 in pool environments were not known in these outbreaks. It is known, however, that acute respiratory and eye symptoms may occur among recreational swimmers in relation to measured levels of NCl_3 in pool environments [3]and NCl_3 is considered to be the causative agent.

Only few and inconclusive studies have been performed on lung function among adults after exposures to measured levels of NCl_3 in pool environments [4,5] and additional studies are required.

Clara cell protein 16 (CC16) is an epithelial protective protein in peripheral lung tissue and changes in its serum levels are used as a biomarker of epithelial integrity [6]. It has been shown to be decreased in relation to frequency of pool attendance [7]. However, changes in serum levels of CC16 have not been studied after short term exposure to NCl_3.

Thickett et al 2002 [8] reported three cases of occupational asthma among British pool workers exposed to NCl_3. There is a lack of epidemiological studies on asthma among those working in swimming pool environments.

The objectives of the present study were 1. To perform a controlled human exposure study of

lung function and biomarkers of pulmonary epithelial integrity in volunteers before and after exposure to indoor swimming pool environments. 2. To perform an epidemiological study of self-reported asthma and subjective symptoms in a cohort of indoor swimming pool workers

DESIGN, MATERIALS AND METHODS

Air sampling and determination of NCl_3:

Exposure measurements in human exposure study

In the two pool environments where our study of volunteers and pool-workers took place hypochlorite was used as disinfectant. Air samples were collected in the breathing zone: one sample for each 2-hour exposure, in total 51 samples.

Determination of NCl_3 at other indoor swimming pools:

Additional determinations of NCl_3 were performed 2004-2008 at 10 different pool establishments (7 conventional ones and 3 “adventure water lands”) in northern Sweden with totally 30 indoor pools. Hypochlorite was used as disinfectant. At each swimming-pool, air was sampled during 3 hours at 3 to 4 different locations in close vicinity of the pool. The equipment was mounted on a stand with the filter at a height of approximately 1.5 meter. Sampling was performed on three different days during winter and three different days during summer.

Air collection and Analysis: 1L/min of air was pumped through a filter (quartz filter QM-A 37 mm Whatman International Ltd., Maidstone, England). The filter was soaked in a solution of sodium carbonate and arsenic trioxide (AsO_3) and dried as presented earlier [9]. When

NCl_3 is collected on the filter it is reduced to chloride ion (Cl^-) [9]. After sampling, the filters were extracted with 10 ml of ultra-pure water, shaken for 30 minutes and filtered through a 13 mm syringe filter (IC Acrodisc®, PALL). The chlorides were analyzed in a suppressed ion chromatography system (Triatlon 900 autosampler, Spark, The Netherlands); ICSep AN1, Anion column (CETAC, Omaha, USA); SCX membrane suppressor column (Sequant, Umeå, Sweden); JD-21 conductivity detector (Costech Microanalytical Ltd, Tallin, Estonia)). The eluent was 7.5 mM NaOH and the suppressor 5 mM H_2SO_4. Control samples of two known chloride concentrations (0.5, 3.0 $mg \cdot l^{-1}$) and at least two blanks were run together with the samples in each run. The chloride concentrations in the blanks were subtracted from the concentration in the samples. The detection limits of NCl_3 (1.78 and 1.18 $\mu g \cdot m^{-3}$ for 2 h and 3 h samplings, respectively) were determined as three times the mean standard deviation of the amount collected on filters of 10 blanks. The limits of quantification (5.9 $\mu g \cdot m^{-3}$ and 3.9 $\mu g \cdot m^{-3}$ for 2 h and 3 h samplings respectively) were determined as ten times the mean standard deviation for the same blanks.

Human exposure study

Study groups:

Group A: 37 healthy subjects (20 men and 17 women, mean age 24.5 years). They were not regular swimming pool visitors and they had not visited a swimming pool within four weeks before study start.

Group B: 14 workers at swimming pools (5 men, 9 women, mean age 39.9 years).

All participants were non-smokers with normal lung function and had no history of allergy or pre-existing lung disease. Subjects were free of airway infection for ≥4 weeks prior to the first exposure and throughout the remainder of the study.

Study design

The study was conducted in a crossover control fashion. Each volunteer was exposed to filtered air in an exposure chamber and on another occasion to an indoor pool environment. In the exposure chamber, located in a separate building away from swimming-pools, incoming air was adjusted to room temperature and filtered through a particle filter. The exposures were performed in random order. Successive exposures were separated by ≥2weeks. The exposures were performed either between 8AM and 10 AM or between 10 Am and 12 AM. All exposures (pool environment or filtered air) lasted for 2 hours. The study subject was exercising on a bicycle ergometer with moderate exercise (minute ventilation 20 $L \cdot min^{-1} \cdot m^{-2}$), during 15-minutes followed by 15 minutes of rest, i.e. four periods of exercise and four periods of rest.

Lung function:

FVC and forced expiratory volume in 1 sec (FEV_1) was determined using a portable spirometer connected to a computer (KoKo Spirometer and KoKo DigiDoser; Pulmonary Data Service Instrumentation, Inc, Louisville, KY, USA), calibrated in the morning and after every 10^{th} measurement. $FEV_{\%}$ was calculated as a percentage of FVC ($FEV_{\%}=FEV_1 x100/FVC$). Lung function was measured immediately before and after exposure in a room with non-detectable levels of NCl_3 (< 0.002 mg NCl_3/m^3) or in a room adjacent to the exposure chamber.

Blood sampling and determination of biomarkers.

We obtained blood samples from the antecubital vein at 0 h and 2 h, i.e. before and after

exposure, and at 4, 6 and 8 hours. Peripheral blood was collected into BD Vacutainer tubes (BD, Plymouth, UK). Each sample was allowed to clot for 1-2 h at room temperature, centrifuged at 3,000x*g* and serum was transferred to cryotubes and frozen at –80°C. These samples were sent to the Industrial Toxicology Unit at the Catholic University of Louvain in Brussels (IUTUCL), Belgium for determination of Clara Cell protein 16 (CC16) and Surfactant Protein D (SPD). CC16 was determined by latex immunoassay using a rabbit anti-CC16 antibody (Dakopatts, Glostrup, Denmark) and CC16 purified at (IUTUCL) as standards [10,11]. All samples were run in duplicate at two different dilutions. The between- and within-run coefficients of variation range 5–10% and results are comparable with ELISA methods [11]. SPD determinations were performed using the Biovendor ELISA kit (Biovendor, Heidelberg, Germany). Analyses were done in duplicate as recommended by the manufacturer.

Total IgE was determined in human serum by a double antibody sandwich ELISA method (Human IgE ELISA kit, Immunology Consultants Lab; Inc, Newberg, OR). The quantity of IgE in the samples was interpolated from a standard curve.

Statistical analyses

All data from CC16 measurements were corrected for diurnal variation according to Helleday et al 2006 [12] and recalculated to correspond to 7 AM. $CC16(corr) = CC16 + 0.582*T - 0.032*T^2$. T is the time after 7.00 AM when the blood sample was taken. Because CC16 values are highest in the morning [12], corrected CC16 values were somewhat greater than measured values.

Statistics: We used repeated measures analyses of variance (Huynh-Feldt corrected) with time and exposure as within-subject factors and group as between-subject factor. Paired t-test or

Wilcoxon signed rank test was used when comparing exposures to filtered air and pool environment at baseline (0 hrs) and after exercise (2h). Median IgE values were compared by the Westenberg-Mood median test. SPSS version 17.0 was used to perform the statistical analyses. A p-value of 0.05 was considered statistically significant.

Epidemiological study

Population:

The epidemiological study group included 1741 persons in the Swedish Census of Population and Housing 1990 who had indicated that they worked at swimming pools. Early 2007 a questionnaire was mailed to them. There was one reminder.

Questionnaire: Questions dealt with: year hired as a pool worker, time periods in various jobs, time spent in swimming pool environments, various symptoms from the respiratory tract and mucous membranes of the eyes and possible use of medication for asthma 589 women and 513 men, age 30 ->80 years (mean age 51.2 years,SD 12.0) responded (63 %). Among 50 non-responders, interviews were performed via telephone. There was a lower prevalence of asthma and respiratory symptoms among the non-responders, not statistically significant. ”Self reported asthma” was derived from a positive answer to the following question: “Do you suffer from asthma or have you suffered from asthma?” Whether a person´s asthma started before or after he/she was hired as a pool worker was derived from the combination of questions about year hired as pool worker and when the first symptoms of asthma occurred.Under the general heading “Acute symptoms when working in a swimming-pool environment” there was a question “How large a part of a working day did you usually spend in the swimming pool environment Hours”

In a nested case-control study within this cohort, 44 cases of self reported asthma occurred after the person was hired as a pool worker. 128 age and sex matched controls were selected within the cohort (mean age 50.5 years SD 10.7).

Exposure assessment:

Based on information on work titles given by each individual, exposure was classified into three different categories; 0, 1, or 2. 0 stands for no exposure, 1 for low exposure and 2 for high exposure. The exposure level is not an estimate of the concentration of NCl_3 in air but is based on the average time during a workday the individual spent in the pool area. Those within category 0 did not spend any time in a pool area, e.g. a cashier. A person within category 1 did occasionally spend some time in the pool area. A manager of a swimming pool or a technician belongs to this category. Individuals belonging to category 2 were those spending most of the workday in the pool area, e.g. a swimming teacher, or a swimming pool worker.

Statistics

Fisher´s test was used to test differences between proportions. Conditional logistic regression was used for analyses in the nested case-control study and logistic regression for analyses of asthma in relation to years worked in swimming-pool environments. All statistical analyses were performed using the statistical package R, version 2.9.0 (www.r-project.org). P-values equal to or less than 0.05 were considered statistically significant.

Ethics

The project was approved by the Regional ethical review board in Umea, Sweden (Dnr 05-044M) and volunteers provided written informed consent. The study was carried out according to the declaration of Helsinki.

RESULTS

Air sampling.

Experimental exposure (Human exposure study)

The NCl_3 levels during the experimental exposures were

Group A: Mean 0.23 mg/m^3 (SD 0.09)

Group B Mean 0.15 mg/m^3 (SD 0.04)

Other Swimming pools

NCl_3 concentrations in air at the 10 different indoor swimming pool establishments were between 0.001- 0.77 mg/m^3, median 0.18 mg/m^3, arithmetic mean (AM) 0.21 mg/m^3 (n=129). The AM concentrations of NCl_3 in each of the ten different pool establishments were between 0.09 – 0.32 mg/m^3. There was no difference in NCl_3 concentrations during summer compared with winter conditions (results not shown).

Human exposure study

Lung function

Group A:

Measured FEV_1 volumes among healthy volunteers as well as the difference before and after 2 hours of exposure to pool environment or filtered air are summarized in Table 1. There was

a small, statistically significant decrease (p=0.01) in FEV_1 (mean decrease = 0.05 L) after exposure to swimming pool air. After exposure to filtered air there was a slight, not statistically significant increase in FEV_1 (mean increase 0.01 L). When comparing the differences (Δ-values) in FEV_1 before and after exposure to pool environment with the Δ-values for exposure to filtered air in the same individuals, the difference between Δ-values was statistically significant (p=0.01).

$FEV_{\%}$ values among healthy volunteers are also given in table 1. After exposure to pool air, there was a small decrease (0.8 $FEV_{\%}$) that was marginally statistically significant (p=0.05). After exposure to filtered air, there was a small (statistically non-significant) increase in $FEV_{\%}$ values. When the individual differences (Δ-values) of $FEV_{\%}$ before and after exposure to pool air were compared with the corresponding Δ-values in filtered air, a statistically significant difference was demonstrated (p=0.004, paired t-test). Airway obstruction is usually defined as $FEV_{\%}$ below 70 (www.goldcopd.com). Only one value was below 70 (after exposure) among the healthy volunteers.

Group B

In table 2, FEV_1 values for the swimming-pool workers are summarized. After exposure to pool air there was a small and not statistically significant decrease in FEV_1, 0.01 L. There was also a small decrease in FEV_1 after exposure to filtered air (0.05 L, p=0.054). When considering the $FEV_{\%}$ values for the workers (Table 2) before and after exposure to pool air, there was a statistically significant decrease of 1.36% (p=0.003). After exposure to filtered air the small decrease in $FEV_{\%}$ of 0.43% was not statistically significant. Only two FEV% values among the pool workers (one before and one after exposure) were below 70. When comparing the Δ-values in filtered air with those in pool air no statistically significant differences were found. The lack of such differences may be partly related to the lower exposure level in group B compared to Group A.

Biomarkers of pulmonary epithelial integrity:

Group A

Mean CC16corr values and related standard deviations (SD) in previously unexposed healthy volunteers, are shown in Figure 1 for 33 of the participants in group A. For the remaining 4 persons, values were missing and they were therefore excluded from analysis.

At baseline (0 hrs), mean CC16corr = 12.6 µg/L before pool exp (0 h) and 10.3 µg/L immediately before (0 h) exposure to filtered air. This difference (p=0.018, paired t-test) is difficult to explain because the same volunteers were exposed to both pool environment and filtered air and they were randomly assigned to either exposure.

Group B

Results are shown in Table 1. The mean CC16corr was 6.5 µg/L before both pool and filtered air exposures.

The difference between groups A and B persisted during and after exposure (0-8 hrs) and is statistically significant ($p<0.001$ repeated measures analysis of variance on log transformed data). There is also a different change with time. Group A decreases with time and group B increases with time. The difference in trend is statistically significant p=0.038.

The decrease with time in group A during and after exposure to pool environment as well as filtered air is statistically significant ($p<0.05$, GLM repeated analysis model). In Group A and Group B there is no statistically significant difference in change with time between pool exposure and filtered air. For improved analysis, values were converted to their natural logarithms, SDs decreased, providing improved statistical conditions, but no statistically significant effect of exposure could be shown (data not shown).

SPD values, shown in Figure 2, also display a change with time, with lower values with increasing time intervals from initiation of exposure.Considering the log transformed SPD variable, there was a difference ($p<0.05$) before and after exposure (i.e. SPD values were higher at 0 hrs than at 2 hrs) and there was a further decrease ($p<0.01$) with time at 2 hrs – 8 hrs (Figure 2). This decrease was similar for exposure to pool air and filtered air. In groups A and B we found no statistically significant changes in SPD values in relation to exposurex.

IgE

The median IgE value was low 1.0 mg/L in Group A and 0.0 in group B.

Epidemiological study

There was a statistically significant relationship between the number of hours, during an average day, spent in the swimming pool environment and the percentage of workers reporting acute symptoms during work ($p<0.01$; logistic regression). Frequent symptoms were: dyspnoea (13%), cough (23%), nose irritation (29%), throat irritation (24%) and eye irritation (37%).

In the nested case-control study, the Odds Ratio (OR) for asthma was 2.53 (95% CI 0.89 – 7.19) for persons with exposure level 2 (114controls,42cases) compared with persons exposed to level 0 or 1 (14 controls, 2 cases). After correction for heredity, the corresponding numbers were: OR 2.31 (95% CI 0.79-6.74).

These values refer to cases of self reported asthma occurring after they started pool work, compared with controls without asthma.

Among individuals who worked more than one year, there was a tendency to a reduced risk of developing asthma in relation to the number of years of work in swimming-pool environments. Only asthma cases that occurred after they started to work as pool workers were considered.. This tendency was, however not, statistically significant p=0.07.

Discussion

Our observations of statistically significant decreases in FEV_1 and $FEV_{\%}$ in previously non-exposed volunteers and in FEV% in pool workers after exposure to pool air are the first such observations in adults.. Carbonelle et al 2002 [4] reported an increase in FEV_1/VC among children and a non-statistically significant decrease in adults (n=13) after they had attended a chlorinated pool. Carbonelle et al 2008 [4] found FEV_1/VC to be unchanged in 11 young adults after swimming in a non-chlorinated pool and slightly, but not statistically significantly decreased after swimming in a chlorinated pool. The lack of statistically significant decrease may be related to the fact that only 11 adults were studied [4], while the statistically significant decrease in our study was based on 37 previously unexposed healthy volunteers.The findings in volunteers were further supported by statistically significant differences in Δ-values. In the 14 pool workers, only one measurement of lung function (FEV%) was statistically significantly decreased and no statistically significant difference was seen when Δ-values were compared. A possible effect in pool workers at the exposure level of our study (0.15mg/m^3) may be considered uncertain.Very few FEV% values were below 70 (indicating no clinically significant airway obstruction within the study group). The reduction in FEV% seen after exposure in pool air here, albeit small, may be a sign of an obstructive airway effect. In children, Bernard et al 2003 [13] found a statistically highly significant relationship between cumulative pool attendance during kindergarten and PEF 15 (post

exercise reduction of peak expiratory flow by 15 percent), providing supportive evidence of airway effects of exposure to chlorinated pool environments.

CC16 levels in serum increase when lung epithelium permeability is adversely affected by air pollutants or other lung toxicants [6, 10,14,15,]. On the other hand, reduced levels of CC16 in lung lavage fluid occur in several lung disorders, probably due to a decrease in the production of CC16 as a consequence of a depletion of Clara cells [16]. We found a statistically significant difference in the serum level of CC16 between pool workers compared to volunteers. This finding is consistent with our previous finding of a lower CC16 value in school children frequently attending indoor swimming pools than in those with a low attendance at such pools [5]. The difference between workers and previously unexposed healthy volunteers may be due to the older age of the workers but is more likely due to repeated exposures because a similar difference occurred among school children and all these differences may be due to a depletion of Clara cells. We did not find any statistically significant exposure-related changes in concentrations of the biomarkers of pulmonary epithelial integrity (CC16 and SPD) after exposure to pool air for 2 hours. The lack of such an exposure-related change was probably due to the relatively short exposure duration and low exposure level of NCl_3. Another possible explanation is that NCl_3 acts preferentially in the more proximal parts of the respiratory tract, inducing a mild constriction of the central airways, but with less interference in the terminal bronchioles, where the Clara cells are located. In previous studies of volunteers exposed to ozone [6], we found both a decrease in FEV_1 and an increase in serum CC16 concentrations after exposure.

Ideally, all exposures should have been performed at the same hour, because it is known that CC16 has diurnal variation [12]. However, for practical reasons exposures were started at

somewhat different times during the day and all CC16 values in the present study were corrected for diurnal variation [12].Such correction is essential, but introduces a certain element of uncertainty. In spite of such correction, there was a statistically significant decrease with time of experiment from 0 h to 8 h in group A (regardless of exposure to NCl_3). This indicates that the real diurnal variation exceeded the one assumed in the employed correction calculation. For group B there is an opposite trend with time, possibly related to an inadequate correction of the values in this group. The pool workers were older and had been more exposed to NCl_3 during many years of work in pool environments. . Our data on SPD, with a statistically significant decrease with time between 0 h and 8 h, confirm previously reported [17] diurnal variation.

The absence of exposure-related effects (after 2 hours exposure) on serum concentrations of CC16 and SPD in combination with small, statistically significant decreases in FEV_1 and $FEV_{\%}$ show that the 2-hour exposure level in this experiment can be regarded as the Lowest-Observed-Adverse-Effect-Level on the lung for this group of volunteers. It should be borne in mind that individuals with increased sensitivity to adverse respiratory effects, like those with pre-existing asthma, were not included in the present study. Our observation may be of use in relation to administrative action in setting exposure limits for NCl_3. To our knowledge, no health-based limit values for occupational or environmental exposures have yet been set for NCl_3. A technical value of 0.2 mg/m^3 was recently recommended in Germany [18].

Bernard et al 2006 [19] showed that serum total IgE was a factor determining the risk of adverse pulmonary effects after exposure to pool environments. Serum levels of total IgE in the volunteers and workers of our study were low. The absence of an increased level of total serum IgE among the present volunteers indicates that individuals with possibly increased sensitivity due to increased IgE had been successfully excluded. Further studies on persons with elevated serum IgE would be of interest. Another group that may suffer respiratory

effects at lower air concentrations of NCl_3 is competitive swimmers because their breathing volumes exceed those of the volunteers in the present study. Helenius et al 1998 [20] found increased respiratory symptoms and bronchial responsiveness in elite swimmers.

Our study indicates that employees in Swedish indoor pools are exposed to approximately the same level of NCl_3 as employees in France and Belgium. We found median NCl_3 concentrations of 0.18 mg/m^3 (mean 0.21 mg/m^3) in ten different premises, while Hery et al 1995 [9] reported 0.14-0.91 mg/m^3 and Massin et al [3] reported a mean of 0.24 mg/m^3 in Public pool environments and 0.67 mg/m^3 in establishments with private owners. There are no previous published data on NCl_3 exposure in Swedish indoor pools. The work environment, i.e. ventilation and the use of sodium hypochlorite as disinfectant has probably not changed during the past decades. This makes it reasonable to estimate that pool workers have been exposed to NCl_3 at approximately the same levels as reported in this study.

In the epidemiological part of the present study, we found a statistically significant relationship between the number of hours spent in swimming pool environments and the percentage of workers reporting acute symptoms when working. The percentage varied from 13 percent for dyspnoea to 37 percent for eye irritation. These findings are in accordance with previous observations in France [3] and Holland [1]. These are subjective symptoms reported in a questionnaire also collecting exposure information and there is a possibility for recall bias. However similar clear outcomes have been reported also in other studies [1,3].

. Our nested case-referent study found an Odds Ratio (OR) for asthma of 2.53 (95% CI 0.89 – 7.19) for workers with more extensive exposure in pool areas (exposure level 2 compared to persons with exposure level 0 or 1). After correction for heredity the corresponding numbers

were: OR 2.31 (95% CI 0.79 - 6.74). These values refer to cases of self-reported asthma occurring after they started to work in swimming-pool environments, compared to controls without asthma.

Cases of asthma in pool workers have been reported in the United Kingdom [8], but no epidemiological evidence has been reported. The findings of the present study did not reach statistical significance and provide only limited support for a causal relationship between asthma and work at indoor swimming pools Individuals who are fit for these type of jobs tend to exercise more regularly and may notice respiratory symptoms; this may contribute to confounding..The fact that there was a tendency towards a decreasing risk of asthma in workers with longer work history may indicate a healthy worker effect due to the irritating properties of NCl_3 in pool environments. A recent study [21] reported a higher prevalence (4.5%) of new-onset asthma among recreational swimmers with >320 hours of cumulative pool attendance compared to 0.4% among swimmers with <320 hours of pool attendance, thus supporting a role for exposure at chlorinated pools for development of asthma. In children engaged in recreational swimming, a statistically significant relationship was shown between cumulative attendance at indoor swimming pools and the probability of developing asthma in those with increased total IgE in serum [13,19]. Attendance at chlorinated pools before the age of 2 years increased the risk of bronchiolitis and asthma [22]

The present findings support the previously advanced hypothesis [7, 13, 19,21] that exposures to NCl_3 levels commonly occurring in indoor swimming pool environments can cause acute airway and mucosal symptoms as well as changes in lung function and deterioration of asthma.

Conclusions: For the first time in adults, statistically significant but small decreases in lung function were found in previously unexposed subjects after exposure to pool air containing 0.23 mg/m^3 of NCl_3 compared to filtered air. The changes in lung function occurred in adults without any signs of allergy and with low IgE values. In a cohort of pool workers we found exposure-related acute mucous membrane and respiratory symptoms. An increased odds ratio for asthma (OR 2.31, 95% CI 0.79-6.74) was indicated in workers in the highest exposure category compared to lower exposures. Our observations give support to a previously advanced hypothesis that current exposures to NCl_3 can cause adverse effects on mucous membranes and lungs of humans and contribute to the development of asthma. Further research in sensitive groups is warranted.

Data sharing: There is no additional data available

Acknowledgements: This work was supported by the Swedish Council for Working Life and Social Research (FAS) project 2004-0497 and the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS) project 2005-1561. The authors declare no competing interests.

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Competing Interests Statement

There are no competing interests.

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Figures and Tables **Table 1.** Healthy volunteers(n=37): FEV_1 (forced expiratory volume, liter during 1 sec) and $FEV_{\%}$ (FEV_1x100/forced vital capacity) measured before and after 2h exercise in filtered air and pool air respectively. Mean ± SD. Mean differences (before-after) within parentheses.

[Separate file]

Table 2. Swimming pool workers (n=14): FEV_1 (forced expiratory volume, liter during 1 sec) and $FEV_{\%}$ (FEV_1x100/forced vital capacity) measured before and after 2h exercise in filtered air and pool air respectively. Mean ± SD. Mean differences (before-after) within parentheses.

[Separate file]

Figure legends

Figure 1:Mean values (µg/L) and SD for CC16corr at various time points before (0h), immediately after exposure (2h) and the following 2 (4h), 4 (6h) and 6 hours (8h). Values are shown for the previously unexposed group of healthy volunteers (A) after exposure in a pool environment, after exposure to filtered air (two upper set of lines and bars). The two lower lines and related bars represent exposure in pool environment and filtered air for Group B, recruited among pool workers with several years exposure to pool environments**.**

Figure 2: Mean and SD for measured SPD values (µg/L) at various time points (0-8 hours) of the study. Exposure to pool environment or filtered air took place for 2 hours (between 0h and 2h). Group A: previously unexposed healthy volunteers. Group B: pool workers

Fig 1 (separate file)

Fig 2(separate file)

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"The contributions of the authors are as follows: G.Nordberg 1,2,3; N-G.Lundstrom 1,2,3; B.Forsberg1,2,3; A.Hagenbjork-Gustafsson1,2,3; B. J-son Lagerkvist1,2,3; J. Nilsson1,2,3: M.Svensson1,2,3; A.Blomberg1, 2,3; L. Nilsson1,2,3; A. Bernard1,2,3; X. Dumont1,2,3; H.Bertilsson1,2,3 and K. Eriksson1,2,3.

1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published."

LUNG FUNCTION and ASTHMA in POOL ENVIRONMENTS

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Lung function in volunteers before and after exposure to trichloramine in indoor pool environments and asthma in a cohort of pool workers

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List of abbreviations:

CC16: Clara Cell protein 16

FEV_1: Forced Expiratory Volume in 1 second, liters

$FEV_{\%}$: FEV_1x100/FVC

FVC: Forced Vital Capacity, liter

NCl_3: Nitrogen trichloride or trichloramine

OR: Odds Ratio

RHINE: Respiratory Health in Northern Europe

SPD: Surfactant protein D

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Summary:

1) Article focus:

Exposure to trichloramine (NCl_3) in swimming-pool air is known to cause mucous membrane and pulmonary effects, but statistically significant changes in lung function among adults have not been reported.

Epidemiological studies of asthma among pool workers are not available.

2) Key messages:

In this study we found for the first time, statistically significant decreases in lung function in volunteers after exposure to pool air with commonly occurring levels of NCl_3.

We found a tendency towards a higher odds ratio (OR) for asthma in a nested case reference study within a cohort of 1102 pool workers.

Our findings support the notion that current workroom exposures of NCl_3 may contribute to asthma development.

3) Strengths and limitations: This is the first study showing small but statistically significant decreases in lung function after exposure to pool air. This is the first nested Case/Control study in pool workers. It reports an OR for asthma of 2.31 (95% CI 0.79-6.74) among pool workers with the highest exposure (after correction for heredity), but this finding did not reach statistical significance.

LUNG FUNCTION and ASTHMA in POOL ENVIRONMENTS

ABSTRACT

Objectives: Exposure to trichloramine (NCl_3) in indoor swimming pool environments is known to cause mucous membrane irritation, but if it gives rise to changes in lung function or asthma in adults is not known. 1: We determined lung function in volunteers before and after exposure to indoor pool environments 2: We studied the occurrence of respiratory symptoms and asthma in a cohort of pool workers.

Design/Methods/Participants: 1. We studied two groups of volunteers, 37 previously non-exposed healthy persons and 14 pool workers, who performed exercise for two hours in an indoor pool environment. NCl_3 in air was measured during pool exposures and in 10 other pool environments. Filtered air exposures were used as controls. Lung function and biomarkers of pulmonary epithelial integrity were measured before and after exposure. 2. We mailed a questionnaire to 1741 persons who indicated in the Swedish census 1990 that they worked at indoor swimming-pools.

Results: 1. In previously non-exposed volunteers, statistically significant decreases in FEV_1 and $FEV_{\%}$ ($p=0.01$ and $p=0.05$ respectively) were found after exposure to pool air (0.23 mg/m^3 of NCl_3). In pool workers, a statistically significant decrease in $FEV_{\%}$ ($p=0.003$) was seen (but no significant change of FEV_1) ~~after exposure to 0.15 mg/m^3 of NCl_3~~. In the 10 other pool environments the median NCl_3 concentration was 0.18 mg/m^3. 2. Our nested Case/Control study in pool workers found an OR for asthma of 2.31 (95% CI 0.79-6.74) among those with the highest exposure. Exposure-related acute mucous membrane and respiratory symptoms were also found.

Conclusions: This is the first study in adults showing statistically significant decreases in lung function after exposure to NCl_3. An increased OR for asthma among highly exposed pool workers did not reach statistical significance, but the combined evidence supports the

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notion that current workroom exposures may contribute to asthma development. Further research on sensitive groups is warranted.

INTRODUCTION and OBJECTIVES

Mono-, di- and trichloramines are formed following a reaction between ammonia (NH_3) or other nitrogen containing substances present in swimming pool water when hypochlorite is used as a disinfectant. Trichloramine (NCl_3) is the most volatile chloramine and is emitted into the air of indoor swimming pools. Exposure to this substance was the suspected cause of outbreaks of short-incubation ocular and respiratory illness [1,2], but concentrations of NCl_3 in pool environments were not known in these outbreaks. It is known, however, that acute respiratory and eye symptoms may occur among recreational swimmers in relation to measured levels of NCl_3 in pool environments [3]and NCl_3 is considered to be the causative agent.

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Only few and inconclusive studies have been performed on lung function among adults after exposures to measured levels of NCl_3 in pool environments [4,5] and additional studies are required.

Clara cell protein 16 (CC16) is an epithelial protective protein in peripheral lung tissue and changes in its serum levels are used as a biomarker of epithelial integrity [6]. It has been shown to be decreased in relation to frequency of pool attendance [7]. However, changes in serum levels of CC16 have not been studied after short term exposure to NCl_3.

Thickett et al 2002 [8] reported three cases of occupational asthma among British pool workers exposed to NCl_3. There is a lack of epidemiological studies on asthma among those working in swimming pool environments.

The objectives of the present study were 1. To perform a controlled human exposure study of lung function and biomarkers of pulmonary epithelial integrity in volunteers before and after exposure to indoor swimming pool environments. 2. To perform an epidemiological study of self-reported asthma and subjective symptoms in a cohort of indoor swimming pool workers

DESIGN, MATERIALS AND METHODS

Air sampling and determination of NCl_3:

Exposure measurements in human exposure study

In the two pool environments where our study of volunteers and pool-workers took place hypochlorite was used as disinfectant. Air samples were collected in the breathing zone: one sample for each 2-hour exposure, in total 51 samples.

Determination of NCl_3 at other indoor swimming pools:

Additional determinations of NCl_3 were performed 2004-2008 at 10 different pool establishments (7 conventional ones and 3 “adventure water lands”) in northern Sweden with totally 30 indoor pools. Hypochlorite was used as disinfectant. At each swimming-pool, air was sampled during 3 hours at 3 to 4 different locations in close vicinity of the pool. The equipment was mounted on a stand with the filter at a height of approximately 1.5 meter. Sampling was performed on three different days during winter and three different days during summer.

Air collection and Analysis: 1L/min of air was pumped through a filter (quartz filter QM-A 37 mm Whatman International Ltd., Maidstone, England). The filter was soaked in a solution of sodium carbonate and arsenic trioxide (AsO_3) and dried as presented earlier [9]. When NCl_3 is collected on the filter it is reduced to chloride ion (Cl^-) [9]. After sampling, the filters were extracted with 10 ml of ultra-pure water, shaken for 30 minutes and filtered through a 13 mm syringe filter (IC Acrodisc®, PALL). The chlorides were analyzed in a suppressed ion chromatography system (Triatlon 900 autosampler, Spark, The Netherlands); ICSep AN1, Anion column (CETAC, Omaha, USA); SCX membrane suppressor column (Sequant, Umeå, Sweden); JD-21 conductivity detector (Costech Microanalytical Ltd, Tallin, Estonia)). The eluent was 7.5 mM NaOH and the suppressor 5 mM H_2SO_4. Control samples of two known chloride concentrations (0.5, 3.0 $mg \cdot l^{-1}$) and at least two blanks were run together with the samples in each run. The chloride concentrations in the blanks were subtracted from the concentration in the samples. The detection limits of NCl_3 (1.78 and 1.18 $\mu g \cdot m^{-3}$ for 2 h and 3 h samplings, respectively) were determined as three times the mean standard deviation of the amount collected on filters of 10 blanks. The limits of quantification (5.9 $\mu g \cdot m^{-3}$ and 3.9 $\mu g \cdot m^{-3}$ for 2 h and 3 h samplings respectively) were determined as ten times the mean standard deviation for the same blanks.

Human exposure study

Study groups:

Group A: 37 healthy subjects (20 men and 17 women, mean age 24.5 years). They were not regular swimming pool visitors and they had not visited a swimming pool within four weeks before study start.

Group B: 14 workers at swimming pools (5 men, 9 women, mean age 39.9 years).

All participants were non-smokers with normal lung function and had no history of allergy or pre-existing lung disease. Subjects were free of airway infection for ≥4 weeks prior to the first exposure and throughout the remainder of the study.

Study design

The study was conducted in a crossover control fashion. Each volunteer was exposed to filtered air in an exposure chamber and on another occasion to an indoor pool environment. In the exposure chamber, located in a separate building away from swimming-pools, incoming air was adjusted to room temperature and filtered through a particle filter. The exposures were performed in random order. Successive exposures were separated by ≥2weeks. The exposures were performed either between 8AM and 10 AM or between 10 Am and 12 AM. All exposures (pool environment or filtered air) lasted for 2 hours. The study subject was exercising on a bicycle ergometer with moderate exercise (minute ventilation 20 $L \cdot min^{-1} \cdot m^{-2}$), during 15-minutes followed by 15 minutes of rest, i.e. four periods of exercise and four periods of rest.

Lung function:

FVC and forced expiratory volume in 1 sec (FEV_1) was determined using a portable spirometer connected to a computer (KoKo Spirometer and KoKo DigiDoser; Pulmonary Data Service Instrumentation, Inc, Louisville, KY, USA), calibrated in the morning and after every 10th measurement. $FEV_{\%}$ was calculated as a percentage of FVC ($FEV_{\%}=FEV_1 x 100/FVC$). Lung function was measured immediately before and after exposure in a room with non-detectable levels of NCl_3 (< 0.002 mg NCl_3/m^3) or in a room adjacent to the exposure chamber.

Blood sampling and determination of biomarkers.

We obtained blood samples from the antecubital vein at 0 h and 2 h, i.e. before and after exposure, and at 4, 6 and 8 hours. Peripheral blood was collected into BD Vacutainer tubes (BD, Plymouth, UK). Each sample was allowed to clot for 1-2 h at room temperature, centrifuged at 3,000x*g* and serum was transferred to cryotubes and frozen at –80°C. These samples were sent to the Industrial Toxicology Unit at the Catholic University of Louvain in Brussels (IUTUCL), Belgium for determination of Clara Cell protein 16 (CC16) and Surfactant Protein D (SPD). CC16 was determined by latex immunoassay using a rabbit anti-CC16 antibody (Dakopatts, Glostrup, Denmark) and CC16 purified at (IUTUCL) as standards [10,11]. All samples were run in duplicate at two different dilutions. The between- and within-run coefficients of variation range 5–10% and results are comparable with ELISA methods [1~~2~~]. SPD determinations were performed using the Biovendor ELISA kit (Biovendor, Heidelberg, Germany). Analyses were done in duplicate as recommended by the manufacturer.

Total IgE was determined in human serum by a double antibody sandwich ELISA method (Human IgE ELISA kit, Immunology Consultants Lab; Inc, Newberg, OR). The quantity of IgE in the samples was interpolated from a standard curve.

Statistical analyses

All data from CC16 measurements were corrected for diurnal variation according to Helleday et al 2006 [1~~2~~3] and recalculated to correspond to 7 AM. $CC16(corr) = CC16 + 0.582*T - 0.032*T^2$. T is the time after 7.00 AM when the blood sample was taken. Because CC16 values are highest in the morning [1~~2~~3], corrected CC16 values were somewhat greater than measured values.

Statistics: We used repeated measures analyses of variance (Huynh-Feldt corrected) with time and exposure as within-subject factors and group as between-subject factor. Paired t-test or Wilcoxon signed rank test was used when comparing exposures to filtered air and pool environment at baseline (0 hrs) and after exercise (2h). Median IgE values were compared by the Westenberg-Mood median test. SPSS version 17.0 was used to perform the statistical analyses. A p-value of 0.05 was considered statistically significant.

Epidemiological study

Population:

The epidemiological study group included 1741 persons in the Swedish Census of Population and Housing 1990 who had indicated that they worked at swimming pools. Early 2007 a questionnaire was mailed to them. There was one reminder.

Questionnaire: Questions dealt with: year hired as a pool worker, -time periods in various jobs, time spent in swimming pool environments, various symptoms from the respiratory tract and mucous membranes of the eyes and possible use of medication for asthma- 589 women and 513 men, age 30 ->80 years (mean age 51.2 years,SD 12.0) responded (63 %). Among 50 non-responders, interviews were performed via telephone. There was a lower prevalence of asthma and respiratory symptoms among the non-responders,- not statistically significant. ”Self reported asthma” was derived from a positive answer to the following question: “Do you suffer from asthma or have you suffered from asthma?” Whether a person´s asthma started before or after he/she was hired as a pool worker was derived from the combination of questions about year hired as pool worker and when the first symptoms of asthma occurred.Under the general heading “Acute symptoms when working in a swimming-pool

environment" there was a question "How large a part of a working day did you usually spend in the swimming pool environment Hours"

In a nested case-control study within this cohort, 44 cases of self reported asthma occurred after the person was hired as a pool worker. 128 age and sex matched controls were selected within the cohort (mean age 50.5 years SD 10.7).

Exposure assessment:

Based on information on work titles given by each individual, exposure was classified into three different categories; 0, 1, or 2. 0 stands for no exposure, 1 for low exposure and 2 for high exposure. The exposure level is not an estimate of the concentration of NCl_3 in air but is based on the average time during a workday the individual spent in the pool area. Those within category 0 did not spend any time in a pool area, e.g. a cashier. A person within category 1 did occasionally spend some time in the pool area. A manager of a swimming pool or a technician belongs to this category. Individuals belonging to category 2 were those spending most of the workday in the pool area, e.g. a swimming teacher, or a swimming pool worker.

~~***Comparison data***~~

~~We obtained data on asthma in 1990 from the study "Respiratory Health in Northern Europe" (RHINE [14]) via one of the authors of the present paper (BF). As we used the same questions in the present study as in RHINE, it was possible to derive adequate sex and age stratified comparison data up to the age of 55.~~

Statistics

Fisher´s test was used to test differences between proportions. Conditional logistic regression was used for analyses in the nested case-control study and logistic regression for analyses of asthma in relation to years worked in swimming-pool environments. All statistical analyses were performed using the statistical package R, version 2.9.0 (www.r-project.org). P-values equal to or less than 0.05 were considered statistically significant.

Ethics

The project was approved by the Regional ethical review board in Umea, Sweden (Dnr 05-044M) and volunteers provided written informed consent. The study was carried out according to the declaration of Helsinki.

RESULTS

Air sampling.

Experimental exposure (Human exposure study)

The NCl_3 levels during the experimental exposures were

Group A: Mean 0.23 mg/m^3 (SD 0.09)

Group B Mean 0.15 mg/m^3 (SD 0.04)

Other Swimming pools

NCl_3 concentrations in air at the 10 different indoor swimming pool establishments were between 0.001- 0.77 mg/m^3, median 0.18 mg/m^3, arithmetic mean (AM) 0.21 mg/m^3 (n=129). The AM concentrations of NCl_3 in each of the ten different pool establishments were

between 0.09 – 0.32 mg/m^3. There was no difference in NCl_3 concentrations during summer compared with winter conditions (results not shown).

Human exposure study

Lung function

Group A:

Measured FEV_1 volumes among healthy volunteers as well as the difference before and after 2 hours of exposure to pool environment or filtered air are summarized in Table 1. There was a small, statistically significant decrease (p=0.01) in FEV_1 (mean decrease = 0.05 L) after exposure to swimming pool air. After exposure to filtered air there was a slight, not statistically significant increase in FEV_1 (mean increase 0.01 L). When comparing the differences (Δ-values) in FEV_1 before and after exposure to pool environment with the Δ-values for exposure to filtered air in the same individuals, the difference between Δ-values was statistically significant (p=0.01).

$FEV_{\%}$ values among healthy volunteers are also given in table 1. After exposure to pool air, there was a small decrease (0.8 $FEV_{\%}$) that was marginally statistically significant (p=0.05). After exposure to filtered air, there was a small (statistically non-significant) increase in $FEV_{\%}$ values. When the individual differences (Δ-values) of $FEV_{\%}$ before and after exposure to pool air were compared with the corresponding Δ-values in filtered air, a statistically significant difference was demonstrated (p=0.004, paired t-test). Airway obstruction is usually defined as $FEV_{\%}$ below 70 (www.goldcopd.com). Only one value was below 70 (after exposure) among the healthy volunteers.

Group B

In table 2, FEV_1 values for the swimming-pool workers are summarized. After exposure to pool air there was a small and not statistically significant decrease in FEV_1, 0.01 L. There was

also a small decrease in FEV_1 after exposure to filtered air (0.05 L, p=0.054). When considering the $FEV_{\%}$ values for the workers (Table 2) before and after exposure to pool air, there was a statistically significant decrease of 1.36% (p=0.003). After exposure to filtered air the small decrease in $FEV_{\%}$ of 0.43% was not statistically significant. Only two FEV% values among the pool workers (one before and one after exposure) were below 70. When comparing the Δ-values in filtered air with those in pool air no statistically significant differences were found. The lack of such differences may be partly related to the lower exposure level in group B compared to Group A.

Biomarkers of pulmonary epithelial integrity:

Group A

Mean CC16corr values and related standard deviations (SD) in previously unexposed healthy volunteers, are shown in Figure 1 for 33 of the participants in group A. For the remaining 4 persons, values were missing and they were therefore excluded from analysis.

At baseline (0 hrs), mean CC16corr = 12.6 µg/L before pool exp (0 h) and 10.3 µg/L immediately before (0 h) exposure to filtered air. This difference (p=0.018, paired t-test) is difficult to explain because the same volunteers were exposed to both pool environment and filtered air and they were randomly assigned to either exposure.

Group B

Results are shown in Table 1. The mean CC16corr was 6.5 µg/L before both pool and filtered air exposures.

The difference between groups A and B persisted during and after exposure (0-8 hrs) and is statistically significant (p<0.001 repeated measures analysis of variance on log transformed

data). There is also a different change with time. Group A decreases with time and group B increases with time. The difference in trend is statistically significant p=0.038.

The decrease with time in group A during and after exposure to pool environment as well as filtered air is statistically significant ($p<0.05$, GLM repeated analysis model). In Group A and Group B t~~T~~here is no statistically significant difference in change with time between pool exposure and filtered air. For improved analysis, values were converted to their natural logarithms, SDs decreased, providing improved statistical conditions, but no statistically significant effect of exposure could be shown (data not shown).

SPD values, shown in Figure 2, also display a change with time, with lower values with increasing time intervals from initiation of exposure.Considering the log transformed SPD variable, there was a difference ($p<0.05$) before and after exposure (i.e. SPD values were higher at 0 hrs than at 2 hrs) and there was a further decrease ($p<0.01$) with time at 2 hrs – 8 hrs (Figure 2). This decrease was similar for exposure to pool air and filtered air. In groups A and B w~~W~~e found no statistically significant changes in SPD values in relation to exposurex.

IgE

The median IgE value was low 1.0 mg/L in Group A and 0.0 in group B., ~~compared to 3.0 mg/L in mild asthmatics (n=18) participating in another study on influence of general air pollution conducted by one of the authors (BF). Compared to the volunteers in the present study (groups A and B), the median value of the asthmatics was statistically significantly higher (p= 0.002) based on Westenberg-Mood median test.~~

Epidemiological study

LUNG FUNCTION and ASTHMA in POOL ENVIRONMENTS

There was a statistically significant relationship between the number of hours, during an average day, spent in the swimming pool environment and the percentage of workers reporting~~incidence of~~ acute symptoms during work (p<0.01; logistic regression). Frequent symptoms were: dyspnoea (13%), cough (23%), nose irritation (29%), throat irritation (24%) and eye irritation (37%).

~~The prevalence of self reported asthma attacks or medication for asthma was higher (p<0.01; Fisher's test) among swimming pool workers in this study (12.3%) compared with the reference group 8,1% (RHINE 1999). When considering rates (age and sex adjusted) by logistic regression, there was still a higher prevalence among swimming pool workers, but less significant (p=0.11).~~

In the nested case-control study, the Odds Ratio (OR) for asthma was 2.53 (95% CI 0.89 – 7.19) for persons with exposure level 2 (114controls,42cases) compared with persons exposed to level 0 or 1 (14 controls, 2 cases). After correction for heredity, the corresponding numbers were: OR 2.31 (95% CI 0.79-6.74).

These values refer to cases of self reported asthma occurring after they started pool work, compared with controls without asthma.

Among individuals who worked more than one year, there was a~~A~~ tendency to a reduced risk of developing asthma in relation to the number of years of work in swimming-pool environments. ~~was indicated among individuals who worked more than one year and d~~Only asthma cases that occurred~~eveloped asthma~~ after they started to work ~~in~~ as pool workers were considered.~~such environments~~. This tendency was, however not, statistically significant p=0.07.

Discussion

Our observations of statistically significant decreases in FEV_1 and $FEV_{\%}$ in previously non-exposed volunteers and- in FEV% in pool workers after exposure to pool air are the first such observations in adults.-. Carbonelle et al 2002 [4] reported an increase in FEV_1/VC among children and a non-statistically significant decrease in adults (n=13) after they had attended a chlorinated pool. Carbonelle et al 2008 [4] found FEV_1/VC to be unchanged in 11 young adults after swimming in a non-chlorinated pool and slightly, but not statistically significantly decreased after swimming in a chlorinated pool. The lack of statistically significant decrease may be related to the fact that only 11 adults were studied [4], while the statistically significant decrease in our study was based on 37 previously unexposed healthy volunteers. The findings in volunteers were further supported by statistically significant differences in Δ-values. In the 14 pool workers, only one measurement of lung function (FEV%) was statistically significantly decreased and no statistically significant difference was seen when Δ-values were compared. A possible effect in pool workers at the exposure level of our study (0.15mg/m^3) may be considered uncertain. Very few FEV% values were below 70 (indicating no clinically significant airway obstruction within the study group). The reduction in FEV% seen after exposure in pool air here, albeit small, may be a sign of an obstructive airway effect. In children, Bernard et al 2003 [143] found a statistically highly significant relationship between cumulative pool attendance during kindergarten and PEF 15 (post exercise reduction of peak expiratory flow by 15 percent), providing supportive evidence of airway effects of exposure to chlorinated pool environments. —.

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CC16 levels in serum increase when lung epithelium permeability is adversely affected by air pollutants or other lung toxicants [6, 10,14,15,-16]. On the other hand, reduced levels of CC16

in lung lavage fluid occur in several lung disorders, probably due to a decrease in the production of CC16 as a consequence of a depletion of Clara cells [16~~17~~]. We found a statistically significant difference in the serum level of CC16 between pool workers compared to volunteers. This finding is consistent with our previous finding of a lower CC16 value in school children frequently attending indoor swimming pools than in those with a low attendance at such pools [5]. The difference between workers and previously unexposed healthy volunteers may be due to the older age of the workers but is more likely due to repeated exposures because a similar ~~like the~~ difference occurred among school children and all these differences may be due to a depletion of Clara cells. We did not find any statistically significant exposure-related changes in concentrations of the biomarkers of pulmonary epithelial integrity (CC16 and SPD) after exposure to pool air for 2 hours. The lack of such an exposure-related change was probably due to the relatively short exposure duration and low exposure level of NCl_3. Another possible explanation is that NCl_3 acts preferentially in the more proximal parts of the respiratory tract, inducing a mild constriction of the central airways, but with less interference in the terminal bronchioles, where the Clara cells are located. In previous studies of volunteers exposed to ozone [6], we found both a decrease in FEV_1 and an increase in serum CC16 concentrations after exposure.

Ideally, all exposures should have been performed at the same hour, because it is known that CC16 has diurnal variation [12]. However, for practical reasons exposures were started at somewhat different times during the day and a~~A~~ll CC16 values in the present study were corrected for diurnal variation [12].Such correction is essential, but introduces a certain element of uncertainty. In spite of such correction, there was a statistically significant decrease with time of experiment from 0 h to 8 h in group A (regardless of exposure to NCl_3). This indicates that the real diurnal variation exceeded the one assumed in the employed

correction calculation. For group B there is an opposite trend with time, possibly related to an inadequate correction of the values in this group. The pool workers were older and had been more exposed to NCl_3 during many years of work in pool environments. ~~Data on diurnal variation for SPD are not available in the literature~~. Our data on SPD, with a statistically significant decrease with time between 0 h and 8 h, confirm previously reported [17]~~indicate that a~~ diurnal variation ~~exists~~.

The absence of exposure-related effects (after 2 hours exposure) on serum concentrations of CC16 and SPD in combination with small, statistically significant decreases in FEV_1 and $FEV_{\%}$ show that the 2-hour exposure level in this experiment can be regarded as the Lowest-Observed-Adverse-Effect-Level on the lung for this group of volunteers. It should be borne in mind that individuals with increased sensitivity to adverse respiratory effects, like those with pre-existing asthma, were not included in the present study. Our observation may be of use in relation to administrative action in setting exposure limits for NCl_3. To our knowledge, no health-based limit values for occupational or environmental exposures have yet been set for NCl_3. A technical value of 0.2 mg/m^3 was recently recommended in Germany [18]. Bernard et al 2006 [19] showed that serum total IgE was a factor determining the risk of adverse pulmonary effects after exposure to pool environments. Serum levels of total IgE in the volunteers and workers of our study were low~~er than among mild asthmatics~~. The absence of an increased level of total serum IgE among the present volunteers indicates that individuals with possibly increased sensitivity due to increased IgE had been successfully excluded. Further studies on persons with elevated serum IgE would be of interest. Another group that may suffer respiratory effects at lower air concentrations of NCl_3 is competitive swimmers because their breathing volumes exceed those of the volunteers in the present study. Helenius et al 1998 [20] found increased respiratory symptoms and bronchial responsiveness in elite swimmers.

Our study indicates that employees in Swedish indoor pools are exposed to approximately the same level of NCl_3 as employees in France and Belgium. We found median NCl_3 concentrations of 0.18 mg/m^3 (mean 0.21 mg/m^3) in ten different premises, while Hery et al 1995 [9] reported 0.14-0.91 mg/m^3 and Massin et al [3] reported a mean of 0.24 mg/m^3 in Public pool environments and 0.67 mg/m^3 in establishments with private owners. There are no previous published data on NCl_3 exposure in Swedish indoor pools. The work environment, i.e. ventilation and the use of sodium hypochlorite as disinfectant has probably not changed during the past decades. This makes it reasonable to estimate that pool workers have been exposed to NCl_3 at approximately the same levels as reported in this study.

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In the epidemiological part of the present study, we found a statistically significant relationship between the number of hours spent in swimming pool environments and the percentage of workers reporting acute ~~incidence of~~ symptoms when working. The percentage varied~~workers reported a high incidence of respiratory and mucous irritation symptoms~~ from 13 percent for dyspnoea to 37 percent for eye irritation. These findings are in accordance with previous observations in France [3] and Holland [1]. These are subjective symptoms reported in a questionnaire also collecting exposure information and there is a possibility for recall bias. However similar clear outcomes have been reported also in other studies [1,3].

~~This study also found a higher prevalence of self reported asthma in swimming pool workers than in a reference group~~. ~~This difference remained when adjusted for age and sex, but failed to reach statistical significance (p = 0.11).~~ Our nested case-referent study found an Odds Ratio (OR) for asthma of 2.53 (95% CI 0.89 – 7.19) for workers with more extensive exposure in pool areas (exposure level 2 compared to persons with exposure level 0 or 1). After correction

for heredity the corresponding numbers were: OR 2.31 (95% CI 0.79 - 6.74). These values refer to cases of self-reported asthma occurring after they started to work in swimming-pool environments, compared to controls without asthma.

Cases of asthma in pool workers have been reported in the United Kingdom [8], but no epidemiological evidence has been reported. The findings of the present study did not reach statistical significance and provide only limited support for a causal relationship between asthma and work at indoor swimming pools Individuals who are fit for these type of jobs tend to exercise more regularly and may notice respiratory symptoms; this may contribute to confounding. T ~~However, t~~he fact that there was a tendency towards a decreasing risk of asthma in workers with longer work history may indicate a healthy worker effect due to the irritating properties of NCl_3 in pool environments. A recent study [21] reported a higher prevalence (4.5%) of new-onset asthma among recreational swimmers with >320 hours of cumulative pool attendance compared to 0.4% among swimmers with <320 hours of pool attendance, thus supporting a role for exposure at chlorinated pools for development of asthma. In children engaged in recreational swimming, a statistically significant relationship was shown between cumulative attendance at indoor swimming pools and the probability of developing asthma in those with increased total IgE in serum [13~~14~~,19]. Attendance at chlorinated pools before the age of 2 years increased the risk of bronchiolitis and asthma [22]

The present findings support the previously advanced hypothesis [7, 13~~14~~, 19,21] that exposures to NCl_3 levels commonly occurring in indoor swimming pool environments can cause acute airway and mucosal symptoms as well as changes in lung function and deterioration of asthma.

Conclusions: For the first time in adults, statistically significant but small decreases in lung function were found in ~~both~~ previously unexposed subjects ~~and pool workers~~ after exposure to pool air containing 0.23 ~~and 0.14~~ mg/m^3 ~~respectively,~~ of NCl_3 compared to filtered air. The changes in lung function occurred in adults without any signs of allergy and with low IgE values. In a cohort of pool workers we found exposure-related acute mucous membrane and respiratory symptoms. An increased odds ratio for asthma (OR 2.31, 95% CI 0.79-6.74) was indicated in workers in the highest exposure category compared to lower exposures. Our observations give support to a previously advanced hypothesis that current exposures to NCl_3 can cause adverse effects on mucous membranes and lungs of humans and contribute to the development of asthma. Further research in sensitive groups is warranted.

Data sharing: There is no additional data available

Acknowledgements: This work was supported by the Swedish Council for Working Life and Social Research (FAS) project 2004-0497 and the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS) project 2005-1561. The authors declare no competing interests.

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Figures and Tables

Table 1. Healthy volunteers(n=37): FEV_1 (forced expiratory volume, liter during 1 sec) and $FEV_{\%}$ (FEV_1x100/forced vital capacity) measured before and after 2h exercise in filtered air and pool air respectively. Mean + SD. Mean differences (before-after) within parentheses.

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~~**Table 1.** Healthy volunteers (n=37): FEV_1 (forced expiratory volume, liter during 1 sec) and FEV% (FEV_1x100/forced vital capacity) measured before and after 2h exercise in filtered and pool air respectively. Mean ± SD. Mean differences (before-after) within parentheses.~~

~~Expiratory volume~~	~~Exposure in filtered air before~~	~~after~~	~~mean diff Δ values~~	~~Exposure in pool air before~~	~~after~~	~~mean diff Δ values~~
~~FEV_1~~	~~4.10 ± 0.85~~	~~4.11 ± 0.87~~	~~(0.01)°~~	~~4.14 ± 0.87~~	~~4.09 ± 0.86~~	~~(0.05)**~~
~~FEV%~~	~~80.5 ± 5.8~~	~~80.9 ± 5.2~~	~~(0.4)°~~	~~80.7 ± 5.3~~	~~79.9 ± 5.3~~	~~(0.8)*~~

~~**FEV_1 significantly lower after exposure to pool air, p = 0.01~~
~~*FEV% lower after exposure to pool air, p = 0.05~~
~~°difference not statistically significant~~

~~The FEV_1 Δ values were -0.01 liter/sec in filtered air and 0.05 liter/sec in pool air, difference statistically significant, p = 0.01 (paired t test).~~
~~Paired t test of the difference in FEV% Δ value in filtered air (mean -0.4 %) as compared pool s air (mean 0.8 %) was statistically significant, p = 0.004.~~

~~**Table 2.** Swimming pool workers (n=14): FEV_1 (forced expiratory volume, liter during 1 sec) and $FEV_{\%}$ (FEV_1x100/forced vital capacity) measured before and after 2h exercise in filtered air and pool air respectively. Mean ± SD. Mean differences (before-after) within parentheses.~~

~~Expiratory volume~~	~~Exposure in filtered air before~~	~~after~~	~~mean diff Δ values~~	~~Exposure in pool air before~~	~~after~~	~~mean diff Δ values~~

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~~FEV_1~~	~~3.56 ± 0.99 3.51 ± 0.91 (0.05)°~~	~~3.59 ± 0.93 3.57 ± 0.92 (0.014) °~~
~~FEV%~~	~~78.86 ±6.3 78.43 ± 5.42 (0.43)°~~	~~79.1 ± 4.1 77.8 ± 5.1 (1.36)*~~

~~*FEV% lower after exposure to pool air, p = 0.003 (Wilcoxon signed rank test).~~
~~° indicates no statistically significant difference~~

Table 2. Swimming pool workers (n=14): FEV_1 (forced expiratory volume, liter during 1 sec) and $FEV_{\%}$ (FEV_1x100/forced vital capacity) measured before and after 2h exercise in filtered air and pool air respectively. Mean + SD. Mean differences (before-after) within parentheses.

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Figure legends

Figure 1:Mean values (µg/L) and SD for CC16corr at various time points before (0h), immediately after exposure (2h) and the following 2 (4h), 4 (6h) and 6 hours (8h). Values are shown for the previously unexposed group of healthy volunteers (A) after exposure in a pool environment, after exposure to filtered air (two upper set of lines and bars). The two lower lines and related bars represent exposure in pool environment and filtered air for Group B, recruited among pool workers with several years exposure to pool environments.

Figure 2: Mean and SD for measured SPD values (µg/L) at various time points (0-8 hours) of the study. Exposure to pool environment or filtered air took place for 2 hours (between 0h and 2h). Group A: previously unexposed healthy volunteers. Group B: pool workers

Fig 1 (separate file)

Fig 2(separate file)

LUNG FUNCTION and ASTHMA in POOL ENVIRONMENTS

Statement:

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Contributorship statement:
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1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published."

Table 1. Healthy volunteers(n=37): FEV_1 (forced expiratory volume, liter during 1 sec) and $FEV_{\%}$ (FEV_1x100/forced vital capacity) measured before and after 2h exercise in filtered air and pool air respectively. Mean ± SD. Mean differences (before-after) within parentheses.

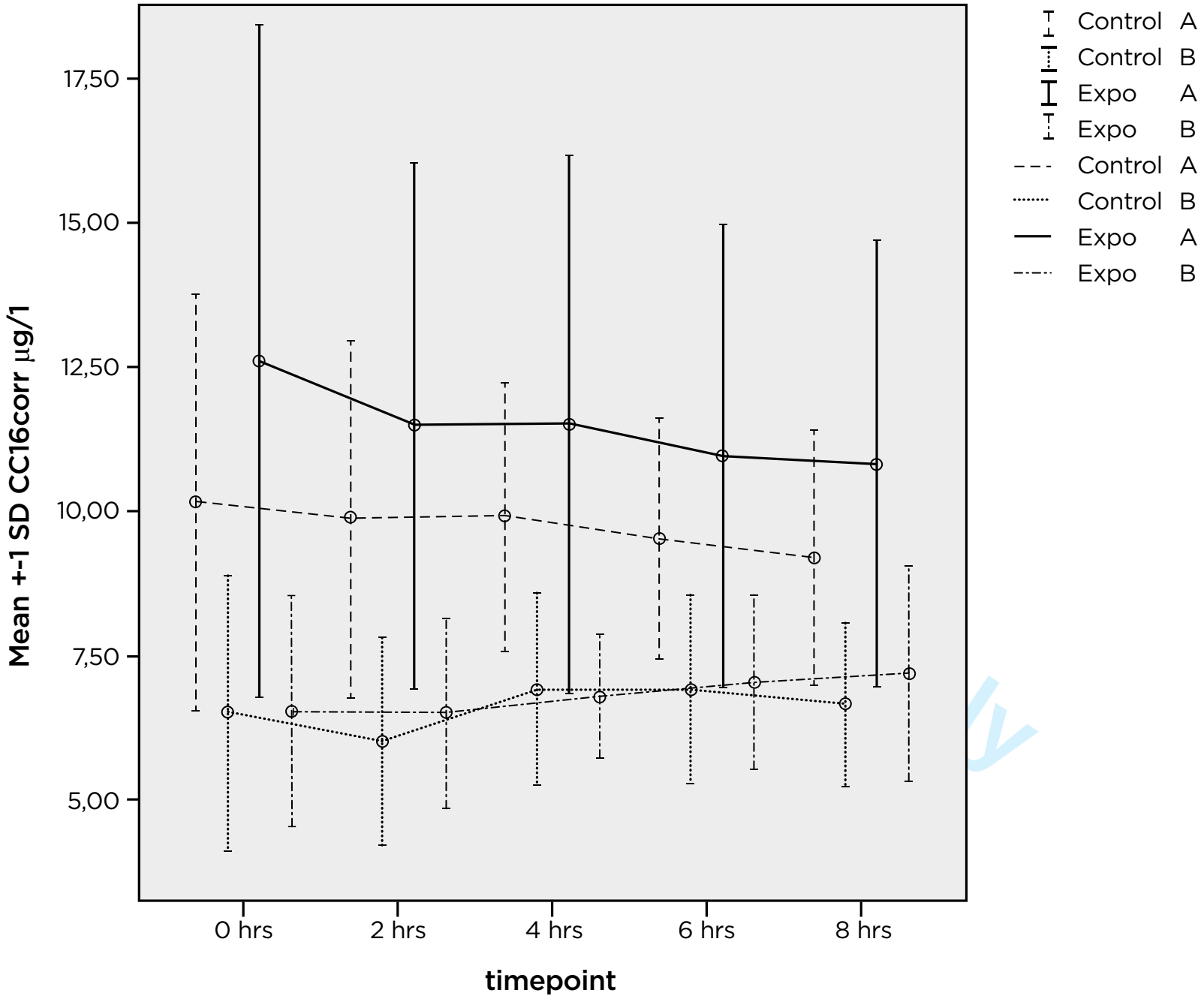
Expiratory volume	Exposure in filtered air before	after	mean diff Δ -values	Exposure in pool air before	after	mean diff Δ -values	Difference in changes≠
FEV_1	4.10± 0.85	4.11 ± 0.87	(-0.01)°	4.14 ± 0.87	4.09 ± 0.86	(0.05) **	p=0.01
FEV%	80.5 ±5.8	80.9 ± 5.2	(-0.4)°	80.7± 5.3	79.9 ± 5.3	(0.8)*	p=0.004

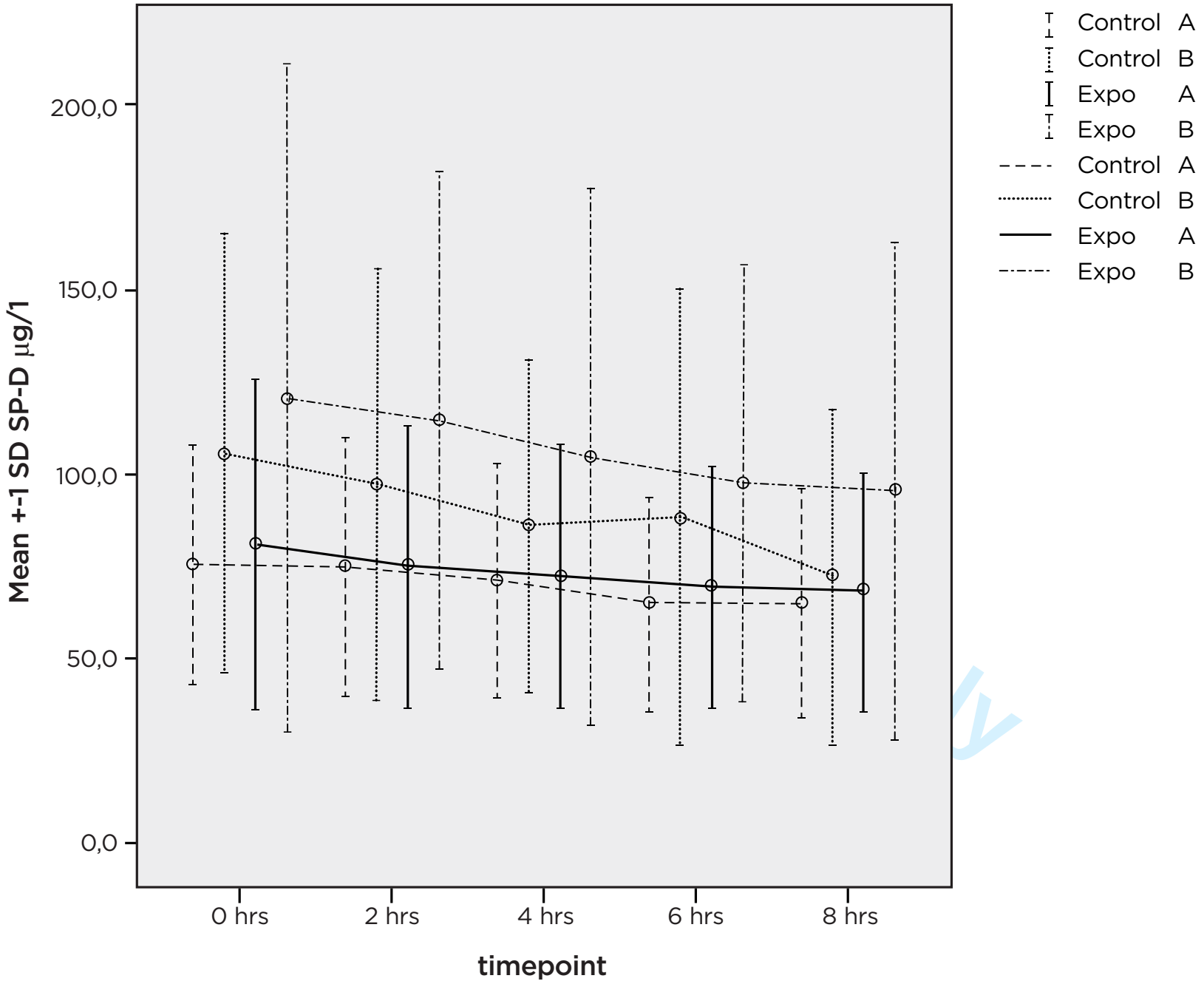
** **FEV_1** significantly lower after exposure in pool air, p=0.01
*FEV% low er after exposure to pool air, p = 0.05
° indicates no statistically significant difference
≠ statistical significance of difference between Δ -values in filtered air and in pool air

Table 2. Swimming pool workers (n=14): FEV_1 (forced expiratory volume, liter during 1 sec) and $FEV_{\%}$ (FEV_1x100/forced vital capacity) measured before and after 2h exercise in filtered air and pool air respectively. Mean ± SD. Mean differences (before-after) within parentheses.

Expiratory volume	Exposure in filtered air before	after	mean diff Δ -values	Exposure in pool air before	after	mean diff Δ -values	Difference in changes[≠]
FEV_1	3.56 ± 0.99	3.51 ± 0.91	(0.05)°	3.59 ± 0.93	3.57 ± 0.92	(0.014) °	Non-significant
FEV%	78.86 ±6.3	78.43 ± 5.42	(0.43)°	79.1 ± 4.1	77.8 ± 5.1	(1.36)*	Non-significant

*FEV% lower after exposure to pool air, p = 0.003 (Wilcoxon signed rank test).
° indicates no statistically significant difference
[≠]Statistical significance of difference between Δ -values in filtered air and pool air


Mean +-1 SD CC16corr µg/1
17,50
15,00
12,50
10,00
7,50
5,00
0 hrs
2 hrs
4 hrs
6 hrs
8 hrs
timepoint
Control A
Control B
Expo A
Expo B
Control A
Control B
Expo A
Expo B


Control A
Control B
Expo A
Expo B
Control A
Control B
Expo A
Expo B
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150,0
100,0
50,0
0,0
Mean +-1 SD SP-D µg/1
0 hrs
2 hrs
4 hrs
6 hrs
8 hrs
timepoint

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported
Objectives	3	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	Present key elements of study design early in the paper
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses

Continued on next page

Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time
		Case-control study—Report numbers in each exposure category, or summary measures of exposure
		Cross-sectional study—Report numbers of outcome events or summary measures
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	18	Summarise key results with reference to study objectives
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.