Pulmonary Epithelial Integrity in Children - Relationship to Ambient Ozone Exposure and Swimming Pool Attendance

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Pulmonary Epithelial Integrity in Children - Relationship to Ambient Ozone Exposure and Swimming Pool Attendance.

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**Key words:** Clara cell protein-CC16, airway irritants, ozone, nitrogen trichloride, swimming pool, children

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**Abbreviations used:**

BMI, Body mass index, kg/m²

EPA, Environmental Protection Agency

FORMAS, Forskningsradet for Miljo, Areella Naringar och Samhalle

O₃, ozone

O₂, oxygen

NO₂, nitrogen dioxide

NCl₃, nitrogen trichloride, trichloramine or chlorine azide

FVC, forced vital capacity, litre

FEV₁, forced expiratory volume in one second, litre/s

FEV₁% predicted, percent of the predicted normal value of FEV₁ calculated from age, sex, height, and weight of the subject
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Abstract

Airway irritants such as ozone ($O_3$) are known to impair lung function and induce airway inflammation. Clara cell protein (CC16) is a small anti-inflammatory protein secreted by the non-ciliated bronchiolar Clara cells. CC16 in serum has been proposed as a non-invasive and sensitive marker of lung epithelial injury. In this study, we used lung function and serum CC16 concentration to examine the pulmonary responses to ambient ozone exposure and swimming pool attendance. The measurements were made on 57 children 10-11 years old before and after outdoor exercise for two hours. Individual ozone exposure was estimated as the total exposure dose between 7 AM until the second blood sample was obtained, (mean $O_3$ concentration/m$^3$ x hours). The maximal one-hour value was 118 $\mu$g/m$^3$ (59 ppb), and the individual exposure dose ranged between 352-914 $\mu$g/m$^3$h. These ozone levels did not cause any significant changes in mean serum CC16 concentrations before or after outdoor exercise. Nor was any decrease in lung function detected. However, children who regularly visited chlorinated indoor swimming pools had significantly lower CC16 levels in serum than non-swimming children both before and after exercise, 5.7±2.4 and 5.3±1.7 $\mu$g/l versus 8.2±2.8 and 8.0±2.6 $\mu$g/l, p<0.002. These results indicate that repeated exposure to chlorination by-products in the air of indoor swimming pools have adverse effects on the Clara cell function in children. A possible relation between such damage to Clara cells and pulmonary morbidity (e.g. asthma) should be further investigated.
**Introduction**

Ozone (O$_3$) is an important component of air pollution. Ground level ozone in urban air is formed in a photochemical reaction between oxygen (O$_2$) and nitrogen dioxide (NO$_2$) from fossil fuel emissions under the influence of sunlight and volatile hydrocarbons. Therefore, the ozone levels tend to be high in areas with high intensity of ultraviolet radiation and high emissions of NO$_2$ from car traffic or industries using fossil fuels (Com Environ Occup Health Ass 1996; de Marco et al. 2002).

Epidemiological and controlled human studies, as well as animal experiments on exposure to ozone have reported airway inflammation and/or a decrease in lung function at ambient concentrations (Balmes 1993, review article; Krishna et al. 1995, review article). Human experimental exposure to ozone has demonstrated a spectrum of acute airway responses. Among these are decrements in forced vital capacity (FVC) and forced expiratory volume in one second (FEV$_1$), increased airway resistance (Blomberg 1999; Seal 1993), altered airway permeability (Koren 1991), and antioxidant defences (Blomberg 1999, Mudway 2001), as well as a neutrophilic airway inflammation (Koren 1991, Schelegle 1991, Stenfors 2002). Most of these studies were short-term exposures on healthy adults. Acute lung function changes in children have been shown in field exposures. Associations between ambient ozone levels and reductions in FVC and FEV$_1$ in children in summer camps and big cities have been shown in several studies (Com Environ Occup Health Ass 1996; Kopp et al. 2000). Recently, short-term effects of ozone were observed in children as an increased frequency of emergency visits for asthma (Fauroux et al. 2000)

Repeated exposure to other environmental and occupational gases, e.g. sulphur dioxide and
chlorine also increase the risk of airway irritation and asthma-like symptoms (Olin et al. 2002). Several studies have shown that competitive swimmers have an increased prevalence of airway inflammation, bronchial hyper-responsiveness, and asthma (Potts 1996; Helenius and Haahtela 2000). This was attributed to inhalation of chlorine gas and its derivatives formed by chlorination of ammonia derived from organic matter in swimming pool water, e.g. NCl3.

In recent years there has been a growing interest in non-invasive indicators as a means to detect early effects of air irritants (Bernard et al. 1992; Broeckaert et al. 1999). Several reports describe different lung-specific secretory proteins, which may be used to detect changes in the number of and/or integrity of epithelial secretory cells (Hermans and Bernard 1999). One of these, CC16 is a small 16 kD protein produced and secreted by the non-ciliated bronchiolar Clara cells, and detectable in serum. CC16 has anti-oxidant properties and the levels in serum increase when lung epithelium permeability is adversely affected by air pollutants or other lung toxicants (Hermans and Bernard 1996; Broeckaert et al. 1999). On the other hand, reduced levels of CC16 in lung lavage fluid are described in several lung disorders, e.g. chronic bronchitis and in smokers. This may be due to a decrease in the production of CC16 depending on a decreased number of Clara cells (Hermans and Bernard, 1999). This study validated CC16 and the lung surfactant proteins A, B, and D in blood as biomarkers of adverse pulmonary effects. The advantage of studying lung proteins, in serum instead of in broncho-alveolar lavage, which has been commonly used to study inflammatory effects in the airways, is obvious. Blood samples are more easily obtained than is lung lavage. Besides, lung lavage is not a suitable method in studies on children.

The general aim of the present program, which was part of a EU project (HELIOS), was to
examine lung function and possible changes in the serum levels of CC16 in relation to ambient ozone exposure in Italy, France, Belgium, and Sweden (cf Bernard 2003). The effects of exposure to other environmental factors, e.g. chlorine and its by-products in swimming pools were also examined. The present study was conducted in Umea, a town in Northern Sweden, with low to moderate ozone levels and was divided in four sub-studies. The first part was conducted on healthy adults in winter, when ozone levels were low, and the second part in the summer when ozone levels were known to be higher than in other seasons. Similar studies were then repeated in children. The present report describes the results from the summer study on children.

**Subjects and Methods**

The winter study on children was conducted in November 2001. 139 children, 63 girls and 76 boys, from four primary schools were recruited. Children with a history of asthma or kidney disease were not invited. The recruitment of school children was done according to the same protocol as in the Bernard et al study in Brussels (Bernard et al. 2003). Lung function measurements were performed and peripheral blood samples were obtained for analysis of CC16 in serum. In the present study 57 of these children, 56 Caucasian and one Chinese, participated. The study was conducted in May 2002. The local Ethics Committee at Umea University approved the study protocol. A written informed consent was obtained from the parents.

The selection of the 57 children (33 boys and 24 girls) from the larger study in November 2001 was based on the results from the lung function tests and a questionnaire answered by the parents. Subjects, who reported pollen allergy, childhood asthma, and/or who had a
forced vital capacity (FVC) or forced expiratory volume in one second (FEV₁) below 80% of the predicted value were not invited. Nor were children selected if there had been some difficulties with the blood sampling, or if the questionnaires were missing. The mean age of the children was 10.8 ± 0.4 years. Lung function testing and blood sampling (like in November 2001) were repeated twice, before and after light exercise outdoors for two hours (range 1.5-3.0 h). The parents completed a questionnaire on e.g. current food intake, passive smoking, and airway illness since the winter study. The participating children answered questions on outdoor activities and swimming pool attendance. Nearly 40% of the children were regular indoor pool visitors (i.e. they had visited an indoor pool for at least one hour per month during six months or longer). Sodium hypochlorite (1% chlorine) was used for the disinfection of the pool water. Based on swimming pool attendance according to the questionnaire, the children were divided into two subgroups, 34 non-pool visitors and 23 pool visitors.

Lung function parameters, e.g. FVC and FEV₁ were determined using a portable spirometer connected to a computerised data program (KoKo Spirometer & KoKo DigiDoser; Pulmonary Data Service Instrumentation, Inc., Louisville, USA). The instruments were calibrated in the morning and after every tenth measurement. Changeable filter mouthpieces were purchased from Intramedic AB, Balsta, Sweden. One trained lung physiologist tested the lung function in all children. The tests were carried out in the standing position. The best of the three best reproducible flow/volume curves was used in the analysis. The computer program calculated the predicted normal values as a function of sex, age, height and weight according to Polgar and Promadhat (1971).

Blood samples were obtained from the antecubital vein after local anaesthesia with a cream
or plaster (EMLA, AstraZeneca Ltd, Sodertalje, Sweden) immediately before (S1; four missing samples) and after the outdoor session (S2; three missing samples). Two CC16 values were available for 20 pool visitors and 31 non-pool visitors. 7.5 ml of blood was drawn in Sarstedt Monovette tubes for serum, Serum Z/9 ml, (Sarstedt, Landskrona, Sweden). Each sample was allowed to clot for 1 – 2 h at room temperature. After centrifugation at 3,000 rpm (within 2 h after sampling), the serum was transferred to cryotubes and frozen at –80° C. These samples were then sent to the Industrial Toxicology Unit at the Catholic University of Louvain in Brussels for analysis. CC16 was determined by a latex immunoassay using rabbit anti-Clara cell protein antibody (Dakopatts, Glostrup, Denmark) and CC16 purified according to the standard in the laboratory (Bernard et al. 1992; Carbonelle et al. 2002). The assay has been validated by comparison with a monoclonal antibody-based enzyme-linked immuno-sorbent assay, ELISA (Hermans et al. 1998). All samples were run in duplicate at two different dilutions. The between- and within-run coefficients of variation ranged from 5-10%.

Outdoor ozone was monitored continuously at the university campus where the children spent the time outdoors, using a Dasibi UV photometry Ozone analyser, model 1108 (Dasibi Environmental Corporation, Glendale, California, US) for these measurements. Ozone exposure was estimated as the total exposure of ozone between seven AM until the second blood sample was taken, between one and four PM (mean O₃ concentration/m³ x number of hours). Since the children spent part of that time indoor (mean four hours), and as it is known from other studies that indoor concentrations of O₃ are lower than outdoors (Com Environ Occup Health 1996), each individual's exposure dose was estimated by assuming an exposure level of 50% of the mean outdoor ozone concentration during time spent indoors. This assessment was confirmed by measurement with passive diffusion.
samplers in the examination room. The filters were purchased from and analysed at the IVL Swedish Environmental Research Institute Ltd in Gothenburg, Sweden. The mean indoor ozone level during the study period was 40 µg/m³ (20 ppb).

The statistical programme SPSS 11 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Differences in FEV1 and CC16 before and after exercise and differences between groups were assessed with Student's $t$-test, paired and unpaired. Pearson correlation tests were used for the analyses of correlations. A p-value <0.05 was considered statistically significant.

Results

The mean daytime outdoor ozone concentration in the days studied ranged from 77 to 116 µg/m³, and the maximal one-hour value was 118 µg/m³. The estimated individual exposure dose varied from 352 to 914 µg/m³-hours.

FEV1 was significantly higher after outdoor exercise than before both in children who had regularly attended chlorinated swimming pools and in children not swimming (Table 1). These differences remained also if the percentages of the predicted FEV1 (FEV1 % predicted) were compared. The mean measured FEV1 values varied between 91.2 - 93.0 percent of the predicted ones. There were no significant differences between pool visitors and non pool visitors, when comparing FEV1 % predicted either before (p = 0.43) or after exercise (p = 0.45, Student’s $t$-test). Neither was there any significant difference in BMI between the two groups of children.
The mean serum concentrations of CC16 in non-pool visitors were 8.2 ± 2.8 µg/l before exercise and 8.0 ± 2.6 µg/l after exercise. The corresponding values in pool visitors were 5.7 ± 2.4 and 5.3 ± 1.7 µg/l (Table 2), range 2.2 - 16.1 µg/l. The BMI was 18.5 ± 2.9 kg/m². Only one pool visitor and three non-visitors were exposed to passive smoke. There were no significant correlations between the serum CC16 levels and parental smoking or BMI. No significant differences were found between pre- and post-exposure levels of serum CC16. Neither, did the time spent outdoors (mean 6 h) during the two days preceding the test day have any influence on the CC16 levels. However, the average CC16 levels in pool visitors both before (S1) and after (S2) exercise were lower than in non-pool visitors, p<0.01 (Table 2). Twenty-two children regularly visited an indoor swimming pool for 1-35 h/month, median 4 h/month. The children had been visiting indoor swimming pools regularly between 6 months to 10 years, median 3 years. Only two children had been swimming since they were babies. No statistically significant relationship to attendance at a swimming-pool during the last days before the test was found, probably because only 7 children had attended indoor swimming pools the last two days before the test. In our study we did not find any correlation between parental smoking and effects on the airways of the children or CC16 levels, possibly because only one pool visitor and three non-pool visitors were exposed to passive smoke.

The correlations between ozone exposure and CC16 levels before or after exercise outdoors were not statistically significant in the group as a whole. However, when CC16 after exercise (S2) was considered, there was a tendency towards a correlation in non-pool visitors after exercise, p < 0.06 (Table 3, Figure 1).
Discussion

In this study, moderate ozone levels between 77 and 116 µg/m³ did not have any adverse effect on the lung function parameter FEV₁ after two hours’ outdoor exercise. In fact, the FEV₁ was slightly increased at the second measurement. This could be an effect of better test performance after exercise than before. The ambient ozone levels in our study are also lower than those reported to affect lung function parameters at ambient ozone concentrations (Kinney et al. 1996; Nickmilder et al. 2003).

The serum CC16 levels found in this study did not correlate with BMI, which has been shown in other studies as well (Hermans et al, 1998). They were of the same magnitude as those recently reported in children of the same age in Belgium (Bernard et al. 2003; Carbonelle et al. 2002). In those studies the serum levels of CC16 in children did not change significantly during swimming exercise. In the present study we have compared the serum CC16 levels in pool visitors and in a control group not exposed to chlorination by-products and found significantly lower levels of serum CC16 in pool visitors suggesting adverse effects on Clara cells.

There were no significant differences between the levels of CC16 before and after outdoor exercise. Neither were there any statistically significant relationships between CC16 levels in serum and ambient ozone exposure, although a marginally significant tendency was found among non-swimmers (Fig 1). The lack of statistical significance may be due to the limited number of subjects and/or the ozone levels not being high enough to cause a response. In the present study the ozone concentration was approximately 1/4 of the ozone concentration which recently was found to increase the serum CC16 levels in adult subjects.
(n = 22) exposed for two hours in an exposure chamber to 400 µg/m³ of O₃ (Blomberg et al. 2003). Another possible explanation for the lack of a clear relationship between serum CC16 and the ozone dose in the present study is that the time period between the measurements was not long enough to cause a measurable change in CC16 levels. There may also be an interference with diurnal variation not corrected for in the present study. Such a diurnal variation was indicated in a recent study on adults, n=19 (Helleday et al. 2003). The reason why Bernard et al. did not find any significant variations in serum CC16 between 9.00 and 10.00 AM to 4.00 and 5.30 PM in seven healthy adults, may be the low number of subjects studied (Bernard et al. 1997).

Lower CC16 levels among subjects regularly attending chlorinated swimming pools are in accordance with the findings by our Belgian partners in the HELIOS project (Carbonelle et al. 2002; Bernard et al. 2003). These authors found that the concentrations of CC16 in trained swimmers were negatively correlated with their cumulated pool attendance. Thus, swimmers seem to have a somewhat decreased pool of CC16 in the Clara cells in the lungs. The CC16 concentration in serum reflects both the epithelial permeability and the integrity of Clara cells (Hermans and Bernard (1999). Therefore, it is conceivable that a repeated exposure to disinfecting by-products formed by hypochlorite and organic matter (e.g. urea and sweat) in pools may decrease the CC16 secretion because of Clara cell dysfunction or damage. Thus, a possible increase in the intra-vascular leakage of CC16 caused by e.g. ozone exposure could be masked by a decrease in the production of CC16 in swimmers (Carbonelle et al. 2002: Bernard et al 2003). That this could be the case also in our study is indicated by the tendency towards a correlation between short-term ozone exposure and the serum CC16 levels in non-pool visitors, but not in pool visitors after exercise.
The levels of chlorination by-products were not measured in this study, but evidently they were high enough to affect the lung epithelium in children regularly visiting indoor pools. As sodium hypochlorite (1% chlorine) was used as a sanitizer of the pool water, increased levels of NCl₃ were likely to be present in the pool air. A limited number of measurements of NCl₃ in indoor air at the swimming pool most frequently used by the swimming children in our study had been performed in 1995. The levels were similar to those reported in the same year from France by Hery et al 1995. They identified NCl₃, as the main component of chlorination by-products present in the air of indoor swimming pool areas (Hery et al. 1995). These authors also reported that symptoms of irritation in the eyes and throat were correlated with the air levels of NCl₃. Bernard et al 2003, report that NCl₃ in public pools typically are in the range 0.1 - 1 mg/m³ in air sampled 1.5 m above the water surface, i.e. similar values as those reported by Hery et al 1995 and in the few Swedish measurements.

Conclusions

Our results indicate that repeated exposure to chlorination by-products in the air of indoor swimming pools have an adverse effect on the Clara cell function in children, so that the anti-inflammatory role of CC16 in the lung could be diminished. A possible role of such influence on Clara cell function in inducing pulmonary morbidity (e.g. asthma) should be further studied. The lung function parameter FEV₁ was not adversely affected by outdoor exercise at a moderate ozone concentration neither in pool visitors, nor in non-pool visitors. A possible effect of ambient ozone on serum CC16 levels (in non-swimming children) needs further investigation.
References


Table 1. Forced expiratory volume during one second (FEV₁, l/s) and % predicted FEV₁ before (S1) and after (S2) outdoor exercise in pool visitors and non visitors, mean and (standard deviation).

<table>
<thead>
<tr>
<th>Category</th>
<th>S1</th>
<th>S2</th>
<th>diff. S2-S1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FEV₁</td>
<td>FEV₁</td>
<td>FEV₁</td>
</tr>
<tr>
<td></td>
<td>FEV₁%pred.</td>
<td>FEV₁%pred.</td>
<td>FEV₁%pred.</td>
</tr>
<tr>
<td>All, N=57</td>
<td>2.19 (0.31)</td>
<td>2.22 (0.32)</td>
<td>0.033 (0.061)</td>
</tr>
<tr>
<td></td>
<td>91.3 (7.2)</td>
<td>92.7 (7.6)</td>
<td>1.4 (2.5)</td>
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<tr>
<td>Non-pool visitors, N=34</td>
<td>2.25 (0.32)</td>
<td>2.29 (0.33)</td>
<td>0.035 (0.063)</td>
</tr>
<tr>
<td></td>
<td>91.2 (5.6)</td>
<td>92.6 (6.3)</td>
<td>1.4 (2.5)</td>
</tr>
<tr>
<td>Pool visitors N=23</td>
<td>2.09 (0.27)</td>
<td>2.13 (0.28)</td>
<td>0.031 (0.060)</td>
</tr>
<tr>
<td></td>
<td>91.5 (9.1)</td>
<td>92.9 (9.5)</td>
<td>1.3 (2.5)</td>
</tr>
</tbody>
</table>
Table 2. CC16 (µg/l) in plasma in children regularly visiting pools and not, before (S1) and after (S2) outdoor exercise, mean ± standard deviation, $\bar{X} \pm SD$.

<table>
<thead>
<tr>
<th>Category</th>
<th>CC16 µg/l (S1)</th>
<th>CC16 µg/l (S2)</th>
<th>Paired t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>All, N=31</td>
<td>7.2 ± 2.9</td>
<td>7.0 ± 2.7</td>
<td>p = 0.31</td>
</tr>
<tr>
<td>Non-pool visitors, N=31</td>
<td>8.2 ± 2.8</td>
<td>8.0 ± 2.6</td>
<td>p = 0.68</td>
</tr>
<tr>
<td>Pool visitors, N=20</td>
<td>5.7 ± 2.4</td>
<td>5.3 ± 1.7</td>
<td>p = 0.14</td>
</tr>
</tbody>
</table>

$t$-test pool visitors $p < 0.002$ $p < 0.001$ versus non-visitors
Table 3. Correlation between individual ozone exposure doses and serum CC16 concentrations in children after exercise (S2).

<table>
<thead>
<tr>
<th>Category</th>
<th>Correlation</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ozone/CC16 (S2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All, N= 54</td>
<td>0.17</td>
<td>p &lt; 0.21</td>
</tr>
<tr>
<td>Non-pool visitors, N=33</td>
<td>0.34</td>
<td>p &lt; 0.06</td>
</tr>
<tr>
<td>Pool visitors, N=21</td>
<td>-0.08</td>
<td>p &lt; 0.74</td>
</tr>
</tbody>
</table>
Figure legend

**Figure 1.** Correlation between the individual ozone exposure dose and serum CC16 concentration (µg/l) after 2 h outdoor exercise. ♦ Non-swimmers ○ Swimmers. The lines represent the correlation presented in table 3 in non-pool visitors — , and in pool visitors - - -
Figure 1.