

Ambient ozone exposure is associated with eosinophil activation in healthy children

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Summary

Background Eosinophil activation is characteristic for allergic airways disease. However, eosinophilic airways inflammation has also been observed subsequent to ambient ozone exposure.

Methods For a population sample of 877 children living at nine sites with different ozone exposure we measured urinary eosinophil protein X (U-EPX) as a marker of eosinophil activation. U-EPX was determined from a single spot urine sample during autumn 1997. Children were participants in a longitudinal study of ozone effects on lung function.

Results The 5–95% percentiles of ozone exposure (30-day mean before test) were 11.8–51.5 p.p.b. (mean: 31.6 ppb). U-EPX was measured by radioimmunoassay and expressed as ratio to urinary creatinine ($\mu\text{g EPX}/\text{mmol creatinine}$). Log transformation was performed to achieve a normal distribution. LogU-EPX was associated with gender, a diagnosis of asthma and atopy (skin test sensitivity to any of seven aeroallergens). LogU-EPX increased with ozone exposure for all children. The medians of LogU-EPX according to the first–fourth quartiles of ozone exposure were: 1.82, 1.88, 1.95 and 2.03. For 172 non-asthmatic children who had spent the whole summer at their site corresponding figures were 1.57, 1.78, 2.07 and 2.13. In a multivariate model with logU-EPX being the dependent variable and adjusted for gender, site and atopy, ozone was found to be significant (estimate: $0.007 \mu\text{g}/\text{mmol creatinine per ppb ozone}$; SE:0.02; $P < 0.001$).

Conclusion Our observation supports the hypothesis that ozone in healthy children is associated with eosinophil inflammation, most likely in the airways.

Keywords: inflammation, ozone, children, epidemiology

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Introduction

Both from experimental studies and field studies there is evidence that ozone at ambient concentrations causes a reduction in lung function. Decrements in volume parameters can be observed in healthy children exercising at ambient ozone below 80 ppb [1]. Whereas it can be argued that these alterations are trivial and not associated with respiratory morbidity, an increase in hospital admissions for

asthma has been observed at similar ozone exposure [1]. Various mechanisms might be involved in the increased susceptibility of asthmatics to the effects of ozone. Ozone might lower lung function in children with already reduced ventilatory function to beneath a critical value where symptoms become more likely. Alternatively, the ongoing inflammation within the airways might be enhanced by ozone. This could happen by an 'intrinsic' pro-inflammatory effect or by a 'priming' effect on the allergen-induced response in allergic asthmatics [2,3]. The airway epithelial cell is the most important target cell in ozone injury [4–6]. The formation of oxygen radicals represents the biochemical

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basis for the subsequent inflammatory events. Ozone reacts slowly with water to give reactive hydroxyl radicals, which are able to oxidize a wide range of bio-molecules. They can diffuse freely into cells and lead to ATP depletion, sulfhydryl oxidation and DNA strand breaks. Glycolysis and mitochondrial respiration are impaired, which may lead to cell death. In places like Mexico City with extremely high chronic ozone exposure epithelial upper airways damage can be observed where atrophy of the nasal mucosa is present in the vast majority of children [7]. *In vitro* data suggest that epithelial cells release pro-inflammatory mediators, such as eicosanoids, cytokines and adhesion molecules which enhance eosinophil chemotaxis and eosinophil adherence to endothelial cells [5]. Our own nasal-lavage studies [8], as well as others [4], have shown that cytotoxic proteins of the eosinophil granulocyte such as ECP are increased in the upper airways after exposure to ozone. It is therefore likely that an enhancement of the eosinophilic inflammation, present in the airways of asthmatics, plays an important role in the impairment of the clinical condition subsequent to ozone exposure. We performed a field-study investigating the health-effects of chronic ozone exposure on healthy school children in Austria, living at different ozone concentrations. The primary endpoint of this study is the development of lung function over a 3-year period [9].

In this analysis we investigated whether eosinophil airway inflammation is associated with ambient ozone in our cohort of children. As a marker of airway inflammation we determined at one survey the concentrations of urinary eosinophil protein X (U-EPX) of all participating children. U-EPX was chosen because [1] it is easy and painless to obtain in children [2], it has been shown that it is associated with the activation of the eosinophil granulocyte as measured by serum ECP or eosinophil protein-X and [3] it is associated with lung function and other markers of disease activity in childhood asthma and other atopic disorders [10–13].

Methods

Study design and subjects

Between September 1997 and October 1997 a cross-sectional study was carried out in a sample of primary school children living at nine sites in lower Austria. Sites with major industry in the vicinity were excluded to reduce confounding by other pollutants. Of the nine sites selected, seven were rural communities with a population size of less than 10 000, and two were towns with a population size of approximately 20 000. All children participated in a longitudinal study on the effects of air pollution on lung function [9,14]. Specifically, the study was set up to test the hypothesis that chronic exposure to ozone reduces lung-

function growth. Hence, study sites were selected to represent a wide range of ozone exposure with other pollutants being as low as possible. Ozone was measured by chemoluminescence technique (Monitor Labs ML8810) according to EPA guidelines. NO₂ was measured using nitric oxide (NO/NO_x) analysers (Monitor Labs 8840, San Diego, CA, USA). SO₂ was measured using fluorescent technique (Monitor Labs 8850). Temperature was continuously monitored at all stations. For this analysis exposure was calculated for each child by taking the mean of all half-hour means collected 30 days (30-day ozone mean) prior to sampling of urine. Children were visited in their schools and spirometry as well as a skin prick test was performed. On the same occasion a urine spot sample was obtained. A self-administered questionnaire was filled out by the parents at home and returned by mail. For the time interval of July and August (summer vacations for primary schools) participants were asked which time period (which weeks) they had spent at their site. Across the nine communities, children stayed at their sites during the summer-time between 71% and 89% (5–95% percentile) of weeks (median 75%). Informed consent was obtained from the parents, and the study protocol was approved by the university's ethical committee.

Urine analyses

Urine was collected from each subject as a spot sample in the schools where all tests were performed. A stick-test was performed to rule out urinary tract infection. All samples were stored at minus 70 °C. Before determination of EPX, urine samples were diluted 11 times in a phosphate buffer containing 0.15 mm NaCl, 1% bovine serum albumin, 0.1% TWEEN20, 10 mm EDTA and 0.2% N-cetyltrimethylammonium-bromide. Urinary EPX was measured by a sensitive and specific commercially available radioimmunoassay (Pharmacia & Upjohn AB, Uppsala, Sweden). Urinary creatinine levels were measured by an Ektachem analyser (Johnson & Johnson Clinical Diagnostics Inc., Rochester, MN, USA). U-EPX concentrations are expressed as µg/mmol creatinine.

Skin prick test

Skin prick testing was performed on a child's left forearm (*Dermatophagoides farinae*, *D. pteronyssinus*, cat, dog, grass-pollen mixture, hazel and birch) including a negative control (0.9% NaCl) and a positive histamine control (10 mg/mL). Wheals were considered positive when the weal size was at least 2 mm and the allergen/histamine ratio at least 0.5. Atopy was defined as at least one positive test to any of the seven allergens.

Table 1. Distribution of 30-days means of NO₂, SO₂ and ozone prior to visit at nine study sites (in ppb)

	Mean	5% percentile	25% percentile	50% percentile	75% percentile	95% percentile
Ozone	31.57	11.77	21.62	32.69	42.23	51.54
NO ₂	7.17	2.02	3.96	5.91	7.91	18.58
SO ₂	2.70	0.86	1.54	2.66	3.64	5.48

Questionnaire

History of respiratory symptoms (12-month period prevalence) suggestive for asthma (wheeze, nocturnal cough, cough after exercise, attacks of breathlessness) as well as a doctor's diagnosis of asthma or recurrent wheezy bronchitis were recorded using a modified ISAAC questionnaire. Recent asthma was defined as a doctor's diagnosis of asthma or recurrent wheezy bronchitis within the last 12 months.

Lung function

Flow volume curves were measured (Jaeger Masterscope, Würzburg, Germany) according to ATS standards. The best of three manoeuvres was used for analyses. For this analysis FEV₁ only was used. A cut-off point of 85% predicted was arbitrarily chosen for stratified analyses of children with/without airways-obstruction. A recent upper respiratory illness was defined as serous/purulent rhinitis on the day where the urine sample was taken.

Statistics

Variables associated with urinary EPX were analysed by linear regression models. Site was considered a random variable and therefore the PROC MIXED (SAS, version

6.06) was used. U-EPX, the variable of main interest, is skewed to the right in its raw condition (before and after the correction to creatinine). Therefore it was log-transformed in order to normalize its distribution. The selection of study sites was made on the basis of a sub-sample of eligible monitor stations. Therefore site was considered random, whereas all other variables on the right side of the equation are assumed to be fixed, which sets up the regressions to mixed models. The statistical analysis was carried out through the SAS package, Version 6.11.

Results

Distributions of exposure data are shown in Table 1 and Fig. 1. Due to the selection of sites a wide range of ozone exposure between sites was observed. The lowest site-specific 30-days ozone exposure before visit was 13.4 ppb (Amstetten) and the highest 51.04 ppb (Wiesmath). A detailed description of the initial population is presented elsewhere [9,14]. For this survey a total of 878 school children (mean age 11.2 ± 0.7 years) were analysed (75.7% of the initial sample of 1150 children). A diagnosis of asthma or recurrent wheezy bronchitis was given in 48 (6.5%) of all children and 114 children reported a history of respiratory symptoms suggestive for asthma. Smoking in the household was reported by 29.8% of all families. Valid lung function values were available for a total of 857

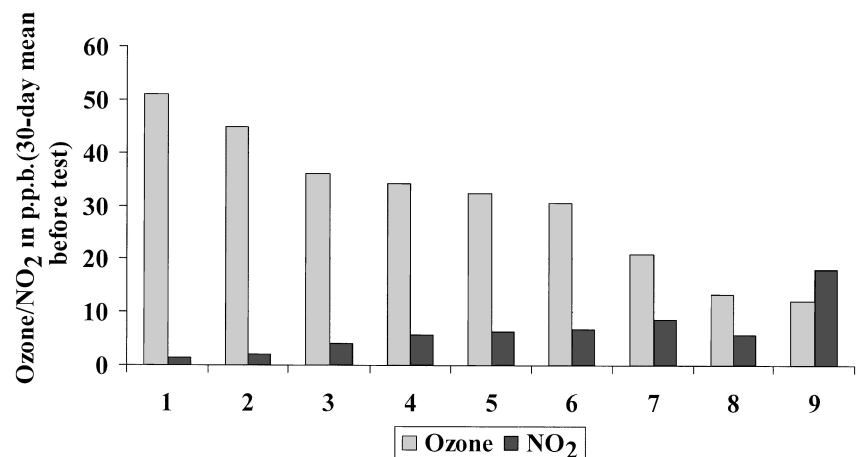


Fig. 1. Ozone/NO₂ means 30 days prior to test at nine study sites.

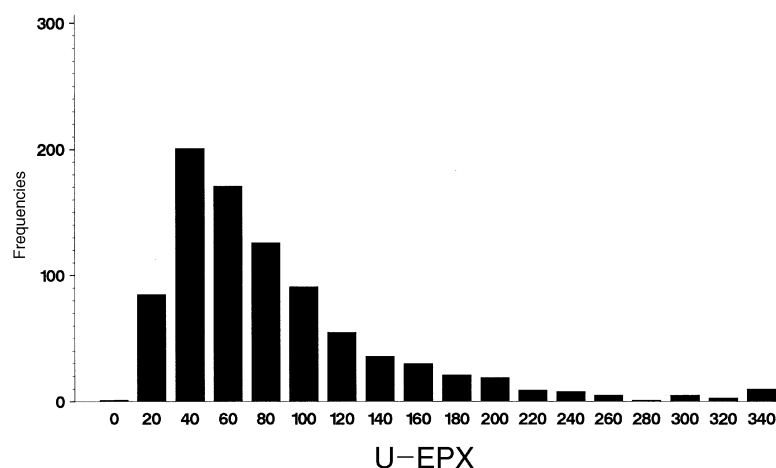


Fig. 2. Distribution of EPX/mcmol creatinine.

children and skin prick data for 784 children. A positive skin test to at least one allergen was observed in 189 (24.1%) of all children tested. Data for one child were removed from analysis because of a recent urinary tract infection with a U-EPX value more than 20-fold increased over mean. Hence, a total of 877 children (505 boys and 372 girls) were studied.

Distribution of U-EPX

Urinary EPX showed a skewed distribution (Fig. 2). Hence, log-transformation was performed. LogU-EPX was related to a doctor's diagnosis of asthma or wheezy bronchitis (mean (SD): 2.2 (0.78) vs. 1.9 (0.66); $P < 0.001$), atopy (2.02 (0.76) vs. 1.89 (0.65); $P < 0.001$) and male gender (2.01 (0.65) vs. 1.83 (0.66); $P < 0.001$). U-EPX increased with ozone exposure for all children. The mean LogU-EPX

values for the first–fourth quartiles of ozone exposure were: 1.82, 1.88, 1.95 and 2.03. No subgroup particularly susceptible to the effects of ozone on LogU-EPX could be delimited (Table 2), the association with ozone being similar in all strata. When analysis was limited to children without current asthma who had spent the whole summer period (July and August) at their site ($n = 172$), the association between U-EPX and ozone increased, with U-EPX concentrations in the fourth quartile being approximately 75% higher as in the first quartile (means of LogU-EPX in the first–fourth quartiles of ozone: 1.57, 1.78, 2.07 and 2.13; Fig. 3). Similar results were obtained when atopic children were excluded from the analysis (data not shown).

Frequency of asthma-specific symptoms was 15.3% for the whole population and was not related to ozone exposure (prevalences of asthma-specific symptoms in the first–fourth quartiles of ozone exposure: 17.4%, 15.6%, 16.8%,

Table 2. Log of urinary eosinophil protein X/creatinine (mean, SD) between subgroups and at quartiles of ozone exposure

		LogU-EPX (mean,SD)	LOG U-EPX at quartiles of ozone exposure (mean of 30 days before lungfunction)			
			0–21.6 ppb	21.6–32.7 ppb	32.7–42.2 ppb	42.2–52.25 ppb
Boys	505	2.01 (0.65) [†]	1.9 (0.65)	1.96 (0.62)	2.01 (0.70)	2.12 (0.70)
Girls	372	1.83 (0.66)	1.71 (0.67)	1.82 (0.60)	1.88 (0.65)	1.89 (0.71)
Atopy +	189	2.02 (0.76) [†]	2.15 (0.73)	2.18 (0.70)	2.27 (0.70)	2.19 (0.71)
Atopy –	595	1.89 (0.65)	1.72 (0.61)	1.80 (0.58)	1.85 (0.63)	1.95 (0.70)
Asthma symptoms +	114	1.98 (0.76)	1.97 (0.83)	1.84 (0.72)	2.19 (0.70)	2.09 (0.80)
Asthma symptoms –	618	1.87 (0.65)	1.77 (0.62)	1.90 (0.61)	1.88 (0.64)	2.00 (0.70)
Asthma +*	48	2.2 (0.78) [†]	2.52 (0.65)	1.5 (0.84)	2.39 (0.79)	2.47 (0.72)
Asthma –	689	1.9 (0.66)	1.77 (0.64)	1.88 (0.61)	1.86 (0.63)	1.99 (0.72)
FEV ₁ < 85% predicted	48	2.03 (0.61)	1.95 (0.28)	1.92 (0.67)	1.95 (0.64)	2.27 (0.67)
FEV ₁ > 85% predicted	809	1.92 (0.67)	1.84 (0.68)	1.90 (0.61)	1.96 (0.66)	2.00 (0.71)
Total	877		1.84 (0.67)	1.90 (0.62)	1.96 (0.65)	2.02 (0.71)

*Doctor's diagnosis of asthma or recurrent wheezy bronchitis, [†] $P < 0.01$.

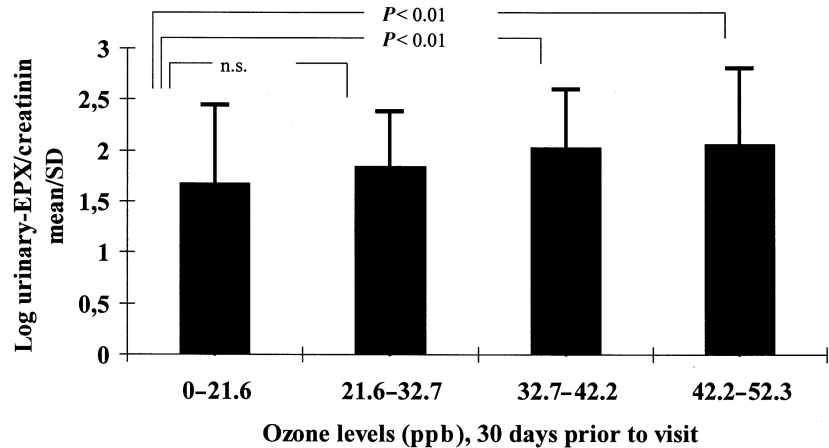


Fig. 3. Quartiles of ozone exposure against log U-EPX/creatinine of non-asthmatic children at home during July and August 1997 ($n = 172$).

12.3%). The frequency of respiratory symptoms within the 48 children with a doctor's diagnosis of asthma or recurrent wheezy bronchitis was not related to ozone exposure. On a bivariate level FEV₁ showed no relationship with ozone. Atopic children were more common in the low ozone stratas (prevalences of atopy in the first–fourth quartiles of ozone exposure: 29%, 25.3%, 24.7%, 18.1%). However, this relationship is driven by a particularly low prevalence (12.6%) at the site with the highest ozone concentration and might therefore represent a random effect.

Multivariate models

In the multivariate model atopy was the strongest predictor for U-EPX. Next to male gender, ozone was significantly related to U-EPX (Table 3). Since lung function could possibly confound the relationship between ozone and U-EPX, analysis was adjusted for FEV₁. Results show that FEV₁ was itself negatively correlated with U-EPX ($\beta = -0.14$; SE 0.06; $P = 0.03$) but did not change the parameter estimate for ozone to a meaningful extent. Since a ratio was chosen as the dependent variable, the ozone effect could be a relationship with either EPX or creatinine. Hence, a model was run with creatinine as an independent variable. In this model no change in the parameter estimate

for ozone was observed. In a model where analysis was restricted to children who were present at their site over the summer period the parameter estimate for ozone was increased approximately twofold. A further model was run adjusting for respiratory symptoms suggestive for asthma. Whereas this variable was significant, the results for ozone were unchanged. Inclusion of temperature into the model did not change the results.

A further polynomial model was introduced to investigate the shape of the dose–response curve which demonstrated no significant effects. The relationship was best described by the linear model. A high correlation was observed between NO₂ and ozone ($r = -0.76$; $P = 0.0001$) leading to a negative parameter estimate when NO₂ was entered into the model ($\beta = -0.01$; SE 0.007; $P = 0.06$). No relationship between SO₂ and U-EPX was observed ($\beta = -10.57$; SE 0.25; n.s.).

Discussion

This is the first epidemiological study to demonstrate an association between ambient ozone and U-EPX, a marker for the activation of the eosinophil granulocyte in a cohort of non-selected healthy young children. On average a 25% increase in U-EPX was observed for all children between

Table 3. Results of multiple linear regression on LogU-EPX/creatinine (adjusted for site as a random variable). $n = 778$

Independent variable	All children			Children at site July–August 1997		
	Estimate	SE	P-value	Estimate	SE	P-value
Gender male	0.16	0.047	0.0007	0.23	0.105	0.03
Atopy +	0.37	0.05	0.0001	0.37	0.13	0.004
Ozone (30-days mean)	0.007	0.0018	0.0001	0.014	0.004	0.0003

the lowest and the highest quartile of ozone exposure. For non-asthmatic children who spent the whole summer period at their site the model predicted an increase of approximately 75% between the lowest and the highest percentile, most likely due to the more precise estimate of exposure. This increase was seen for all strata of children, including strata of children with positive skin tests and children with a diagnosis of asthma or wheezy bronchitis.

It is unlikely that other pollutants have biased our findings. Study sites were selected for ozone exposure and other pollutants were very low. All sites were in a rural vicinity, with major industry or motorways absent. Allergen exposure might vary between sites and we have not implemented pollen counts in our study. However, the relationship between U-EPX and ozone was seen for non-atopic children for whom allergen exposure can be assumed not to be relevant.

Recent controlled exposure studies suggested that ozone might prime the airways for the effects of allergen exposure on bronchial responsiveness [3]. Increased hospitalization rates for asthma were observed in some countries concurrent with peaks in ozone exposure [1]. In our data set, we could not demonstrate that respiratory symptoms in atopic children or in those with a diagnosis of asthma were more likely with increasing exposure. There was no difference in lung function in these groups of children related to ozone exposure, either. However, our study was not set up to test the hypothesis of an increased susceptibility of selected groups to the effects of ozone. Since the primary endpoint was to investigate long-term effects on the general paediatric population we looked at a non-selected sample of healthy children. There were only 48 asthmatics in the study and frequency of positive skin tests were different between the ozone strata. We assume that factors other than ozone (such as allergen exposure) relate to U-EPX in atopic children leading to a higher variability of this marker, thus masking in part the effect of ozone.

Furthermore, respiratory symptoms were asked for retrospectively in a single questionnaire, distributed after the summer period and not prospectively by symptom diaries. Hence, a type two error cannot be ruled out.

Our group [11,15], as well as others [10,13], has shown in clinical studies that the concentration of U-EPX differentiates healthy children from those with atopy or asthma. Its concentration increases with severity and decreases subsequently to appropriate anti-inflammatory treatment. In our study atopy was the strongest predictor for U-EPX, most likely reflecting eosinophil airway inflammation. The negative association with lung function and higher values in boys suggests that U-EPX has a strong relationship with the asthmatic phenotype. However, U-EPX seems to reflect not only allergic airway inflammation, but was shown to be increased in children with cystic

fibrosis [16] or chronic tonsillitis [17]. Activation of the eosinophil has also been shown in children with chronic inflammatory bowel disease [18].

Our data suggest that the eosinophil granulocyte is activated after exposure to ozone at ambient conditions and leads most likely to airway inflammation. In a nasal lavage study we performed previously [8], we have shown, like others [4] by an increase in nasal ECP after exposure, that eosinophils are activated. This was true for non-atopic as well as for atopic children. *In vitro* studies have shown that the airway epithelium releases pro-inflammatory mediators (IL-8; GM-CSF, TNF- α) that increase eosinophil chemotaxis and adhesion to endothelial cells after ozone exposure [5,6]. We do not know whether the activation of eosinophils observed in our study is of any clinical relevance because normal values for U-EPX are lacking and our study design did not allow us to investigate the interaction between allergen exposure and ozone on clinical outcomes. Further studies are needed to investigate in 'true life' whether ozone is additive to allergen exposure in its effect on the eosinophil in the atopic population or alternatively acts as a non-specific pro-inflammatory stimulus.

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References

- 1 Bascom R. State of the art. Health effects of outdoor air pollution (Part I). *Am J Respir Crit Care Med* 1996; 153:3–56.
- 2 Peden DB, Setzer RW Jr, Devlin RB. Ozone exposure has both a priming effect on allergen-induced responses and an intrinsic inflammatory action in the nasal airways of perennially allergic asthmatics. *Am J Respir Crit Care Med* 1995; 151 (5):1336–45.
- 3 Joerres R, Nowak D, Magnusson H. The effect of ozone exposure on allergen responsiveness in subjects with mild asthma or rhinitis. *Am J Respir Crit Care Med* 1996; 153:66–65.
- 4 Hiltermann TJ, de-Brujine CR, Stolk J et al. Effects of photochemical air pollution and allergen exposure on upper respiratory tract inflammation in asthmatics. *Am J Respir Crit Care Med* 1997; 156 (6):1765–72.
- 5 Devalia JL, Bayram H, Rusznak C et al. Mechanisms of pollution-induced airway disease: in vitro studies in the upper and lower airways. *Allergy* 1997; 52 (38 (Suppl.):45–51.

- 6 Leikauf GD, Simpson LG, Santrock J et al. Airway epithelial cell responses to ozone injury. *Environ-Health-Perspect* 1995; 103 (Suppl. 2):91–5.
- 7 Calderon-Garciduenas L, Osorno-Velasquez A, Bravo-Alvarez H, Delgado-Chavez R, Barrios-Marquez R. Histopathologic changes of the nasal mucosa in southwest metropolitan Mexico city inhabitants. *Am J Pathol* 1992; 140:225–32.
- 8 Frischer Th, Kuehr J, Pullwitt A et al. Ambient ozone causes upper airways inflammation in children. *Am Rev Respir Dis* 1993; 148:961–4.
- 9 Frischer Th, Studnicka M, Gartner Ch et al. Lung function growth and ambient ozone. A three years population study in school children. *Am J Respir Crit Care Med* 1999; 160:390–6.
- 10 Venge P, Dahl R, Fredens K, Hällgren R, Peterson C. Eosinophil cationic proteins (ECP and EPX) in health and disease. In: Yoshida T, Torisu M, eds. *Immunobiology of the Eosinophil*. New York: Elsevier Science, 1983:163–78.
- 11 Lugosi E, Halmerbauer G, Frischer T, Koller DY. Urinary eosinophil protein X in relation to disease activity in childhood asthma. *Allergy* 1997; 52:584–8.
- 12 Koller DY, Herouy Y, Götz M et al. Clinical value of monitoring eosinophil activity in asthma. *Arch Dis Child* 1995; 73:413–7.
- 13 Kristjansson S, Strannegard IL, Strannegard O et al. Urinary eosinophil protein X in children with atopic asthma: a useful marker of anti-inflammatory treatment. *J Allergy Clin Immunol* 1996; 97:1179–87.
- 14 Studnicka M, Hackl E, Pischinger J et al. The effects of long term NO₂ on asthma and respiratory symptoms in 7-year olds. *Europ Respir J* 1997; 10:2275–8.
- 15 Halmerbauer G, Frischer Th, Koller DY. Monitoring of disease activity by measurement of inflammatory markers in atopic dermatitis in childhood. *Allergy* 1997; 52:765–9.
- 16 Koller DY, Götz M, Eichler I, Urbanek R. Eosinophil activation in cystic fibrosis. *Thorax* 1994; 49:496–9.
- 17 Mevio E, Perano D, De-Amici M, Maccario R, Monafò V. Chronic tonsillitis in children: activation of polymorphonuclear cells from peripheral blood and tonsillar tissue. In vitro production of MPO, ECP and EPX. *Acta Otolaryngol Stockh* 1996; 523 (Suppl.):101–4.
- 18 Luck W, Becker M, Niggemann B, Wahn U. In vitro release of eosinophil cationic protein from peripheral eosinophils reflects disease activity in childhood Crohn disease and ulcerative colitis. *Eur J Pediatr* 1997; 156 (12):921–4.