

Asthmatic symptoms among pupils in relation to microbial dust exposure in schools in Taiyuan, China

Zhao Z, Sebastian A, Larsson L, Wang Z, Zhang Z, Norbäck D. Asthmatic symptoms among pupils in relation to microbial dust exposure in schools in Taiyuan, China.

Pediatr Allergy Immunol 2008.

© 2008 The Authors

Journal compilation © 2008 Blackwell Munksgaard

Microbial exposure has been indicated as significant in the development of asthma and allergy among children. The aim of the study was to test whether microbial exposure and allergens in the school environment are associated with asthmatic symptoms in pupils. Data on asthmatic symptoms and respiratory infections were collected through a questionnaire survey among 1993 pupils aged 11–15 yr in 10 randomly selected schools in Taiyuan, China. Settled dust in classrooms was analysed using tandem gas chromatography-mass spectrometry for 3-hydroxy fatty acids, marker of lipopolysaccharide (LPS) from endotoxin, muramic acid (MuA), marker of bacteria and ergosterol (Erg) for fungi, quantifying both culturable and non-culturable microbes. A total of 29.8% reported daytime attacks of breathlessness, 8.4% wheeze and 1.2% had doctor's diagnosed asthma. Generally, MuA was negatively associated with wheeze and daytime attacks of breathlessness, the latter of which was negatively associated with Erg to a weaker extent. Total concentration of LPS was positively associated with daytime attacks of breathlessness, but shorter lengths of LPS, C10, C12 and C14 LPS were negatively associated with either wheezing or daytime attacks of breathlessness. For MuA and C10 and C12 of LPS, the associations were independent of airborne allergens and classroom crowdedness, and even independent of the other two microbial markers for MuA. Microbial exposure indicated by certain chemical markers (e.g. MuA) could be protective for asthmatic symptoms, but for LPS (endotoxin), the picture is more complex, varying by different lengths of fatty acids of LPS.

Zhuohui Zhao¹, Aleksandra Sebastian², Lennart Larsson², Zhuanhua Wang³, Zheng Zhang³ and Dan Norbäck¹

¹Department of Occupational and Environmental Medicine, University Hospital and Uppsala University, Uppsala, Sweden, ²Division of Medical Microbiology, Department of Laboratory Medicine, University of Lund, Sweden, ³Institute of Biotechnology, Shanxi University, Taiyuan, China

Key words: allergy; asthma; bacteria; dust; endotoxin; indoor environment; moulds

Zhuohui Zhao, Department of Occupational and Environmental Medicine, University Hospital and Uppsala University, Uppsala SE 751-85, Sweden
Tel.: +46 (0)18 611 3646
Fax: +46 (0)18 51 99 78
E-mail: zhuohui.zhao@medsci.uu.se

Accepted 5 October 2007

There has been an increase of the prevalence rate in asthma and allergy in westernized societies over the past few decades (1, 2). Cumulative evidence, especially from comparative studies in rural areas and developing countries, has suggested that a high prevalence of asthma and allergy is associated with urbanization and Western lifestyles. Among the factors investigated, including allergens, air pollutants, traffic, family sibling size, day-care attendance, breast feeding and diet, microbial exposure is becoming

an increasingly common denominator in explaining the disparity between different areas, even more pronouncing by comparing children living in farm and non-farm environments (3). Evidence on the exposure of micro-organisms has been well reviewed from many different perspectives, in support of the hypothesis that one major factor in the current asthma and atopic epidemic could be the disconnection of humans and the soil, the latter of which acts as a major source of microbial exposure (4).

This is related to the 'hygiene hypothesis', which, however, is undergoing modifications with multiple faces (5, 6). Around 15 yr ago, it was proposed that early life infections and unhygienic conditions ('microbial stimulation') could reduce the risk of allergy (7). Current research has explored the effects of both viral and bacterial infections and of environmental exposure to specific microbial compounds (5). Because of the findings that both viable and non-viable bacterial components are immunologically active, bacterial cell wall components, as well as those for fungi, have sparked a renewed interest in exploring their possible associations with allergy and asthma.

Until now, major cell wall components of bacteria, lipopolysaccharide (LPS, endotoxin) and muramic acid (MuA) are the two most studied compounds for bacteria. LPS is a chemical marker for Gram-negative bacteria. MuA is specific to the bacteria domain but more representative of Gram-positive bacteria than of Gram-negative (8). Fungal cell walls differ from those of bacteria but ergosterol (Erg) is now also being used in experimental sample analysis besides β -glucans (9). The finding that Toll-like receptors (TLR) in innate immunity can recognize conserved microbial structures sheds light on the immunological activity of these microbial components (10). To be brief, TLR4 is considered to be the primary receptor for LPS, TLR2 is the main receptor for Gram-positive bacteria, and both TLR4 and TLR2 can be important for recognition of fungi, of which the process is more complex (11).

Most epidemiological studies on bacterial components have examined endotoxin exposure, but the findings have been contradictory (12). Some demonstrated protective effects of endotoxin, both on atopy and respiratory symptoms (12, 13), while others showed associations with increased asthma symptoms, medication use and degree of airflow obstruction in children and adults with asthma (14, 15). Recently, some research has found that the critical determinants of LPS partly resides in the length and number of acyl (fatty acid) chains of the lipid A moiety in LPS structure, together with the conformation of fatty acid chains (16, 17). It is natural to expect that differentiation of different types of LPS by the fatty acid analysis will better describe the biological effects of LPS, even more pronouncing in an epidemiological way of research. MuA receives relatively less research in this aspect, but a recent publication has shown concentrations of MuA in children's mattress dust were inversely associated with wheeze and asthma, even when

controlling for LPS concentration values (18). The protective effects were also reported by other studies (5, 19). However, there are few studies on environmental exposure to microbial components in Asia (19), where the prevalence of asthma and allergy is still on a low level but with a potentially increasing trend (20).

In this study, we have investigated the associations between microbial exposure and pupils' asthmatic symptoms in Taiyuan, a city in central north China. This was performed in a school environment, as school is one of the most important indoor environment for pupils (21). Three chemical markers of microbial exposure, LPS, MuA and Erg, were analysed in the settled dust in classrooms by tandem gas chromatography-mass spectrometry (GC-MS/MS), a new sensitive and specific method developed especially for the chemical analysis of endotoxin (22). Different types of LPS (endotoxin) were differentiated according to various chain lengths of 3-hydroxy fatty acids (3-OH FA). In addition, allergens in the classrooms were analysed in this study to investigate the associations of microbial exposure independent of allergen contamination.

As far as we know now, very few epidemiological studies on asthma and allergy have applied these new chemical methods to characterize microbial exposure (18, 19). As China has the largest population in the world, epidemiological studies on children's asthma and allergy related to microbial exposure in Chinese schools is and will be of great significance.

Materials and methods

Study population

Ten junior high schools in urban areas of Taiyuan city were arbitrarily selected. The headmasters were contacted and all agreed to participate. The schools had 4–7 first-year classes. If there were five classes or more in the first grade, five classes were representatively selected, to reflect different floors and different parts of the buildings. For schools with four classes, all were selected. A total of 46 first-year classes were included, encompassing 2209 students aged 11–15 yr. The study was approved by the Ethics Committee of the Uppsala University, Sweden.

Information on asthma, asthmatic symptoms and allergy

We used a self-administrated questionnaire to collect data on respiratory health. It was adapted from standardized questions used in International Study of Asthma and Allergies in

Childhood (ISAAC) (23), European Community Respiratory Health Survey (ECRHS) (24) and a previous Swedish and Chinese school study (25). One set of questions was about asthma, including ever asthma, doctor's diagnosed asthma, current asthma attacks and current asthma medication. Other questions were about current airway symptoms without using the word 'asthma', including wheezing or whistling in the chest, daytime attacks of breathlessness during rest and after exercise and nocturnal attacks of breathlessness in the last 12 months. A question about airway infections during the past 3 months was also included. The questionnaire was translated from Swedish to Chinese, and back-translated to Swedish by another person. It was distributed to the pupils in the class 1 wk before the measurements and answered with the help of parents at home.

Classroom inspection and indoor and outdoor climate

Each classroom was inspected by a trained environmental hygiene researcher during December 2004–January 2005. The basic information included the number of students, classroom volume (m^3), type of heating and ventilation system and signs of mould growth or dampness. For indoor climate measurements, three classrooms in each school were selected. Because of practical reasons, 24 classrooms were measured for room temperature ($^{\circ}\text{C}$), relative humidity (RH, %) and CO_2 (p.p.m.) by a direct-reading instrument with an in-built data logger (Q-TrakTM IAQ-monitor; TSI Incorporated, St Paul, MN, USA). Air exchange rate per hour (ac/h) in the classrooms was calculated based on the equilibrium CO_2 concentrations, room volume and number of students (25). Simultaneously, the corresponding outdoor climate was monitored.

Measurements of LPS, MuA and Erg

Totally 78 dust samples were collected from 39 classrooms, while seven classrooms could not be sampled because of lack of electric outlet. Settled dust was collected by a vacuum cleaner of 400 W equipped with a special dust collector fitted with a Millipore filter (pore size $6\text{ }\mu\text{m}$) (ALK Abello, Copenhagen, Denmark). According to the manufacturer, the filters can retain 74% of particles $0.3\text{--}0.5\text{ }\mu\text{m}$, 81% of particles $0.5\text{--}1.0\text{ }\mu\text{m}$ and 95% of particles $1\text{--}10\text{ }\mu\text{m}$. Two samples were collected for each classroom, one from the corridor side and the other from the window side. Vacuum cleaning was

performed for totally 4 min for each sample, 2 min on the floor and 2 min on the desks and chairs. The filters were stored at -20°C until analysis.

The dust samples were sieved (particle diameter $<400\text{ }\mu\text{m}$) and 1–5 mg portions of the fine dust fraction were used for analyses of MuA, Erg and 3-OH FA with 10, 12, 14, 16 and 18 carbon chain lengths by GC-MS/MS (22). A hydrolysate of ^{13}C -labelled cyanobacterial cells was used as internal standards for the analysis of MuA and 3-OH FA, and dehydrocholesterol was used for the analysis of Erg. Briefly, MuA was analysed as methyl ester *o*-methyl acetate derivative after acid methanolysis of samples followed by removal of lipid material. Erg was analysed as a trimethylsilyl (TMS) derivative, after saponification of dust samples, followed by extractions and purification using silica gel column. 3-OH FA were analysed as methyl ester TMS derivatives after strong acid methanolysis, extraction and silica gel column purification. Total LPS concentration was calculated from the sum of the concentration of C10, C12, C14 and C16, divided by a factor of four, as each LPS molecule has four molecules of 3-OH FA (26). C18 was excluded from the LPS calculation as a result of the poor correlation of C18 level by this method and the classical *Limulus* Amebocyte Lysate test (9). The microbial amount per sample for different microbial markers was calculated by multiplying the concentration by the total weight of the dust sample.

Measurements of allergens in settled dust and airborne dust

The same samples were used to analyse the allergen levels using enzyme-linked immunosorbent assay, including cat (Fel d 1), dog (Can f 1), horse (Equ c x), house dust mite (Der p 1 and Der f 1) and cockroach (Bla g 2) allergens. In addition, airborne dusts were collected simultaneously and used to analyse the allergen levels. Detailed information on sample collection and analysis has been published previously (27).

Statistical methods

The differences of prevalence in respiratory health between boys and girls and pupils with and without parental asthma or allergy were tested by chi-square test. For classes with dust collection, we used arithmetic mean values of the two dust samples to represent the class level; for the seven classes without dust collection, we addressed the school mean value to the classes as variation between classrooms within schools

was less than the variations between schools (data available on request). Associations between environmental microbial exposure and symptoms were examined by multiple logistic regression, controlling for age, gender, parental allergy or asthma, environmental tobacco smoke (ETS) at home and other selected home environmental variables. Mutual adjustment was performed among concentrations of three microbial components. Correlations between microbial components, pupil crowdedness in classrooms and allergen levels were analysed using the Kendall Tau- β rank correlation test. Odds ratio (OR) with 95% confidence interval (95% CI) was calculated for all regression analyses. All statistics were performed with the Statistical Package for the Social Sciences (SPSS) 12.0.1, using two-tailed tests at a 5% significance level.

Results

Of the 2209 pupils who received the questionnaire, 1993 participated (90.2%). Girls accounted for 49.3%, and the average age was 13 yr old (s.d. = 0.64, range 11–15 yr).

Pupils' respiratory health, parental disease and selected home environment

According to the questionnaire survey, ever asthma, doctor's diagnosed asthma and current asthma were not common (all <2%) and only 3.8% of surveyed pupils reported furry pet or

pollen allergy (Table 1). On the other hand, respiratory symptoms, especially daytime attacks of breathlessness over the past 12 months (29.8%) and respiratory infections over the past 3 months (39.3%), were commonly reported. No significant differences were found between boys and girls except on daytime attacks of breathlessness after exercise, for which girls reported higher than boys (OR 1.62, 95% CI 1.32–1.97, $p < 0.001$).

Parental asthma or allergy was reported by 11.2% of pupils, and was associated with pupils' both cumulative incidence of asthma (ever asthma), asthma medication, wheeze, daytime attacks of breathlessness and a history of own atopy (Table 2). Recent new home painting, new floor material and new home furniture were reported by 14.0%, 6.7% and 36.8% of pupils respectively. In addition, ETS was common in the pupils' home. Around 67% of pupils reported some smoking at home, among which 30.4% reported daily exposure to ETS at home. These home environmental factors were also associated with pupils' respiratory symptoms and thus controlled in the regression model. Detailed effects of the home environment will be published in a separate publication.

Classroom inspection and indoor and outdoor climate

On average, there were 48 pupils in each classroom with a mean room volume of 193 m³ (Table 3). No classroom had any mechanical ventilation system, and opening windows was the

Table 1. Prevalence (%) of self-reported asthma, asthmatic symptoms and allergies among pupils in Taiyuan, China

	Total† (n = 1981)	Boys (n = 1005)	Girls (n = 976)	OR (95% CI)
<i>Asthma</i>				
Ever asthma	1.8	2.1	1.4	0.68 (0.34–1.34)
Doctor's diagnosed asthma	1.2	1.2	1.1	0.95 (0.42–2.16)
Current asthma attacks	0.4	0.5	0.3	0.62 (0.15–2.60)
Current asthma medication	0.5	0.4	0.6	1.56 (0.44–5.53)
<i>Current airway symptoms in the last 12 months</i>				
Wheezing or whistling in the chest	8.4	9.6	7.2	0.73 (0.53–1.00)
Daytime attacks of breathlessness‡	29.8	25.8	34.0	1.50 (1.24–1.83)*
Daytime attacks of breathlessness at rest	5.4	4.6	6.1	1.37 (0.92–2.03)
Daytime attacks of breathlessness after exercise	27.7	23.4	32.4	1.62 (1.32–1.97)*
Nocturnal attacks of breathlessness	2.1	1.8	2.5	1.39 (0.75–2.58)
At least one airway symptom§	33.9	32.7	37.9	1.45 (1.20–1.75)*
A history of atopy¶	3.8	3.2	4.3	1.40 (0.87–2.23)
Respiratory infections (in the last 3 months)	39.3	39.9	38.8	0.96 (0.80–1.15)

* $p < 0.001$.

†Twelve missing on question of gender.

‡Calculated as daytime attacks of breathlessness either at rest or after exercise.

§Wheezing or whistling in the chest, daytime or nocturnal attacks of breathlessness.

¶Self-reported furry pet or pollen allergy.

OR, odds ratio; CI, confidence interval.

Table 2. Prevalence (%) of self-reported asthma, asthmatic symptoms and allergies among pupils in Taiyuan, China with and without parental asthma or allergy (n = 1993)

	Parental asthma or allergy		OR (95% CI)
	Yes (n = 224)	No (n = 1769)	
<i>Asthma</i>			
Ever asthma	4.5	1.4	3.30 (1.56–6.98)*
Doctor's diagnosed asthma	1.3	1.1	1.19 (0.35–4.03)
Current asthma attacks	0.9	0.3	2.66 (0.53–13.3)
Current asthma medication	1.8	0.3	5.36 (1.50–19.2)*
<i>Current airway symptoms in the last 12 months</i>			
Wheezing or whistling in the chest	15.2	7.6	2.19 (1.46–3.29)**
Daytime attacks of breathlessness†	43.3	28.0	1.93 (1.45–2.56)**
Daytime attacks of breathlessness at rest	10.3	4.7	2.26 (1.39–3.67)*
Daytime attacks of breathlessness after exercise	38.4	26.3	1.77 (1.32–2.38)**
Nocturnal attacks of breathlessness	2.7	2.0	1.34 (0.56–3.22)
At least one airway symptom‡	32.1	48.2	2.02 (1.51–2.70)**
A history of atopy§	6.7	3.4	1.92 (1.07–3.45)***
Respiratory infections (in the last 3 months)	37.1	39.6	0.90 (0.67–1.20)

*p < 0.01; **p < 0.001; ***p < 0.05.

†Calculated as daytime attacks of breathlessness either at rest or after exercise.

‡Wheezing or whistling in the chest, daytime or nocturnal attacks of breathlessness.

§Self-reported furry pet or pollen allergy.

OR, odds ratio; CI, confidence interval.

Table 3. Average indoor and outdoor climate and ventilation in 10 schools in Taiyuan, China during the measuring period

	n*	M (s.d.)	Min–Max
<i>Indoor climate and ventilation</i>			
No. of students	46	48 (8)	33–60
Classroom volume (m ³)	46	193 (18)	161–225
CO ₂ (p.p.m.)	24	2211 (1005)	789–4170
Air exchange rate (ac/h)	24	2.86 (1.85)	0.91–7.32
Temperature (°C)	24	14.7 (2.2)	11.2–18.4
Relative humidity (%)	24	42 (10)	31–62
Settled dust weight per sample (g)	39	1.12 (0.69)	0.12–3.50
<i>Outdoor climate</i>	n‡	M (s.d.)	Min–Max
CO ₂ (p.p.m.)	10	522 (26)	480–559
Temperature (°C)	10	–1.8 (2.1)	–5.5–2.6
Relative humidity (%)	10	52 (11)	30–64

*No. classrooms for indoor climate.

‡No. schools for outdoor climate.

M (s.d.), arithmetic mean (standard deviation).

only way to ventilate. The mean CO₂ concentration was 2211 p.p.m. (789–4170 p.p.m.). Most floors consisted of bare concrete without any paint, in a few cases with tiles but with no wall-to-wall carpeting. The average room temperature was 14.7°C and the RH level was <45%. There

were no visible signs of mould or damp spots in any classroom.

Microbial components in the settled dust

Measurements of both concentration and amount per sample values for three microbial chemical markers are presented in Table 4. For 3-OH FA, higher chain lengths of 3-OH FA were found in higher concentrations. Next, mutual correlations within three different microbial markers were investigated (data not shown). Concentration of MuA was correlated with concentrations of LPS (Tau beta 0.40, p < 0.01) and Erg (Tau beta 0.24, p < 0.01), whereas no significant correlation found between LPS and Erg (Tau beta 0.13, p = 0.23). There were no significant correlations between dust weight and concentration of any microbial marker, but strong correlations existed within three microbial markers in the amount per sample values, e.g. between MuA and LPS (Tau beta 0.71, p < 0.001), between MuA and Erg (Tau beta 0.69, p < 0.001) and between LPS and Erg (Tau beta 0.59, p < 0.001).

Taiyuan is one of the most polluted areas in China, with annual mean PM₁₀ of 252 µg/m³ and winter means of SO₂ and NO₂ of 700 and 52 µg/m³ respectively (Zhao, *et al*, unpublished data). In this project, we also measured SO₂ and NO₂ inside and outside of schools; such data has been published in a PhD. thesis by Zhao (28). There were no significant correlations between the total dust amount or any of the microbial exposures and indoor concentrations of SO₂ and NO₂.

Associations between microbial exposure and asthmatic symptoms

Initially, associations between microbial components and respiratory health were analysed in a regression model after controlling for age, gender, parental asthma or allergy, ETS and new painting, new floor materials and new furniture at home, by keeping each microbial exposure factor separate (Table 5). Current asthma attacks and asthma medication were not analysed because of low prevalence (<1%).

Results showed that wheeze and daytime attacks of breathlessness were negatively associated with MuA concentration, the latter of which was also negatively associated with Erg. A history of atopy was negatively associated with Erg. No associations were found for nocturnal attacks of breathlessness and respiratory infections. Cumulative incidence of asthma and doctor's diagnosed asthma were not associated

Table 4. Average values of microbial components in the settled dust across 39 classrooms in Taiyuan, China

	Concentration			Amount per sample		
	M (s.d.)	GM (g.s.d.)	Min–max	M (s.d.)	GM (g.s.d.)	Min–max
MuA*	9.53 (4.60)	8.55 (1.62)	2.10–24.56	11.39 (8.80)	7.31 (3.16)	0.72–35.63
Erg*	0.69 (0.40)	0.57 (1.87)	0.15–1.78	0.71 (0.60)	0.49 (2.53)	0.07–2.35
LPS†,‡	18.64 (6.19)	17.61 (1.42)	7.92–34.69	21.01 (13.15)	15.05 (2.70)	1.39–54.31
C10†	1.70 (1.62)	1.48 (2.07)	0.00–6.87	2.14 (2.49)	1.36 (3.25)	0.00–9.03
C12†	10.00 (4.13)	9.37 (1.42)	4.96–24.45	11.36 (7.80)	8.01 (2.66)	0.90–33.81
C14†	21.47 (6.55)	20.37 (1.41)	7.62–35.20	23.39 (13.84)	17.37 (2.53)	1.56–56.20
C16†	41.39 (18.95)	37.44 (1.58)	15.98–97.95	47.14 (33.76)	31.99 (2.89)	2.97–150.5
C18†	35.52 (13.05)	34.28 (1.57)	13.25–63.05	40.61 (28.07)	1.36 (3.25)	2.52–123.2

*The concentration values are in the unit of $\mu\text{g/g}$ dust and the amount values in unit of $\mu\text{g/sample}$.

†The concentration values are in the unit of nmol/g dust and the amount values in unit of nmol/sample .

‡LPS is calculated by $(\text{C10} + \text{C12} + \text{C14} + \text{C16})/4$ as one mole of LPS molecules carrying four moles of 3-OH FA.

M (s.d.), arithmetic mean (standard deviation); GM (g.s.d.), geometric mean (geometric standard deviation); LPS, lipopolysaccharide, 3-OH FA, 3-hydroxy fatty acids.

with any microbial exposure (data not presented).

As a next step, we performed mutual adjustment for concentration values by including all three compounds in the model simultaneously (Table 6). The ORs for remaining associations were mainly unchanged. A new significant positive association turned up between LPS concentration and daytime attacks of breathlessness. Amounts of microbial compounds were not possible to analyse by mutual adjustment, because of high inter-correlation between each other.

Furthermore, we investigated effects of different types of 3-OH FA in both concentration and amount values (Table 7). Generally, shorter lengths of LPS tended to be negatively while longer lengths tending to be positively associated with asthmatic symptoms. For wheeze, significantly negative associations were found

only with C10 LPS. Daytime attacks of breathlessness were negatively associated with C10 and C12 as well as the amount of C14. Respiratory infections were negatively associated with concentration of C12 LPS. In this model, however, no associations were found between nocturnal attacks of breathlessness and any microbial component, either by concentration or amount values.

Associations between allergens and asthmatic symptoms

Almost no detectable allergens were found in the settled dust. However, in airborne dust samples, cat allergen (Fel d 1) and dog (Can f 1) were detected and present at a relatively high level of $16.8 \text{ ng/m}^2/\text{day}$ and $17.7 \text{ ng/m}^2/\text{day}$ (geometric mean) respectively. Detailed data and discussion can be found in the publication by Zhao et al.

Table 5. Associations (OR with 95% CI) between microbial components or airborne allergens and respiratory health effects among pupils ($n = 1993$) in Taiyuan, China†

	Wheezing or whistling in the chest	Daytime attacks of breathlessness	Nocturnal attacks of breathlessness	A history of atopy	Respiratory infections (last 3 months)
Concentration‡					
MuA	0.60 (0.39–0.94)*	0.69 (0.54–0.88)**	0.57 (0.23–1.40)	0.73 (0.40–1.34)	0.85 (0.69–1.86)
Erg	0.87 (0.55–1.40)	0.71 (0.53–0.94)*	1.34 (0.57–3.17)	0.47 (0.23–0.98)*	1.25 (0.97–1.62)*
LPS	1.01 (0.76–1.35)	1.07 (0.90–1.28)	1.09 (0.63–1.89)	1.02 (0.68–1.53)	1.00 (0.85–1.18)
Amount per sample§					
MuA	0.81 (0.64–1.01)	0.80 (0.70–0.91)**	0.63 (0.39–1.04)	0.82 (0.59–1.12)	0.94 (0.84–1.06)
Erg	0.77 (0.55–1.07)	0.66 (0.54–0.81)***	0.69 (0.34–1.38)	0.57 (0.34–0.95)*	0.93 (0.79–1.11)
LPS	0.94 (0.81–1.09)	0.92 (0.84–1.00)*	0.81 (0.61–1.08)	0.96 (0.78–1.18)	1.00 (0.93–1.09)*
Airborne allergens					
Cat (Fel d1)	1.02 (0.71–1.44)	0.94 (0.76–1.17)	0.70 (0.33–1.49)	1.28 (0.83–1.99)	1.07 (0.88–1.30)*
Dog (Can f 1)	1.24 (0.72–2.11)	1.13 (0.81–1.57)	0.70 (0.22–2.20)	1.33 (0.63–2.80)	0.99 (0.72–1.36)

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

†Controlling for age, gender, parental asthma or allergy, ETS at home, recent home painting, new floor and new furniture in the past 12 months.

‡OR was calculated for concentrations of LPS, MuA and Erg with an increase of 10 nmol/g , $10 \mu\text{g/g}$ and $1 \mu\text{g/g}$ dust respectively.

§OR was calculated for amount per sample of LPS, MuA and Erg with an increase of 10 nmol/sample , $10 \mu\text{g/sample}$ and $1 \mu\text{g/sample}$ respectively.

OR, odds ratio; CI, confidence interval; MuA, muramic acid; LPS, lipopolysaccharide; Erg, ergosterol.

Table 6. Associations (OR with 95% CI)† between concentrations of microbial components and respiratory health among pupils (n = 1993)‡

	Wheezing or whistling in the chest	Daytime attacks of breathlessness	Nocturnal attacks of breathlessness	A history of atopy	Respiratory infections (last 3 months)
MuA (10 µg/g)	0.49 (0.29–0.85)*	0.62 (0.46–0.84)**	0.38 (0.12–1.15)	0.81 (0.37–1.75)	0.74 (0.57–0.96)*
Erg (µg/g)	1.06 (0.66–1.71)	0.82 (0.61–1.11)	1.63 (0.69–3.80)	0.50 (0.23–1.09)	1.40 (1.06–1.84)*
LPS (10 nmol/g)	1.27 (0.91–1.76)	1.27 (1.04–1.54)*	1.45 (0.76–2.76)	1.13 (0.72–1.76)	1.09 (0.91–1.31)

*p < 0.05; **p < 0.01.

†OR was calculated for concentrations of LPS, MuA and Erg with an increase of 10 nmol/g, 10 µg/g and 1 µg/g dust respectively.

‡Mutually adjusted for three microbial components, after controlling for age, gender, parental asthma or allergy, ETS at home, recent home painting, new floor and new furniture in the past 12 months simultaneously.

OR, odds ratio; CI, confidence interval; LPS, lipopolysaccharide; Erg, ergosterol; MuA, muramic acid.

Table 7. Associations (OR with 95% CI) between each type of LPS and respiratory health effects among pupils (n = 1993) in Taiyuan, China†

	Wheezing or whistling in the chest	Daytime attacks of breathlessness	Nocturnal attacks of breathlessness	A history of atopy	Respiratory infections (last 3 months)
Concentrations (10 nmol/g)					
C10	0.14 (0.04–0.51)**	0.39 (0.20–0.78)**	0.07 (0.01–1.10)	0.36 (0.06–2.06)	0.78 (0.42–1.73)
C12	0.67 (0.41–1.08)	0.75 (0.68–0.99)*	0.58 (0.20–1.67)	0.84 (0.44–1.60)	0.78 (0.61–0.99)*
C14	1.11 (0.85–1.46)	1.01 (0.86–1.20)	1.27 (0.75–2.16)	0.93 (0.63–1.37)	0.99 (0.86–1.17)
C16	1.02 (0.93–1.11)	1.05 (0.99–1.10)	1.04 (0.87–1.23)	1.03 (0.91–1.17)	1.01 (0.96–1.17)
C18	1.10 (0.96–1.25)	1.03 (0.95–1.11)	1.01 (0.78–1.30)	1.13 (0.94–1.35)	1.02 (0.95–1.10)
Amount per sample (10 nmol/sample)					
C10	0.31 (0.13–0.71)**	0.52 (0.33–0.81)**	0.22 (0.04–1.30)	0.60 (0.20–1.81)	0.84 (0.57–1.25)
C12	0.82 (0.64–1.03)	0.80 (0.69–0.92)**	0.62 (0.37–1.04)	0.86 (0.62–1.20)	0.90 (0.79–1.02)
C14	0.95 (0.84–1.09)	0.91 (0.84–0.98)*	0.84 (0.65–1.09)	0.93 (0.78–1.12)	0.97 (0.90–1.04)
C16	0.99 (0.94–1.05)	0.99 (0.96–1.02)	0.96 (0.87–1.07)	1.00 (0.93–1.08)	1.01 (0.98–1.04)
C18	0.99 (0.94–1.07)	0.97 (0.94–1.00)	0.93 (0.82–1.06)	1.01 (0.92–1.10)	1.01 (0.97–1.04)

*p < 0.05; **p < 0.01; ***p < 0.001.

†Controlling for age, gender, parental asthma or allergy, ETS at home, recent home painting, new floor and new furniture in the past 12 months.

LPS, lipopolysaccharide; OR, odds ratio; CI, confidence interval.

(27). In this study, using the same regression model, no association was found between any airborne allergen and pupils' respiratory health (Table 5).

Correlations between microbial components, crowdedness and allergens

Using Kendall Tau- β rank correlation test (Table 8), three microbial components and different types of LPS were correlated with pupil crowdedness in classrooms and airborne cat allergen (Fel d 1). This correlation was much more pronounced in microbial values by amount per sample. However, when we further re-tested the associations between microbial components and pupils' respiratory symptoms by adding these two possible confounding factors, there were no big changes in most of the remaining associations for wheeze and daytime attacks of breathlessness (data not shown).

Discussion

We found associations between current respiratory symptoms in junior high school pupils and microbial components in classroom dust in this area of China. Both MuA, a marker of bacteria, and Erg, a marker of fungi, seemed to be protective, while the effect of endotoxin (LPS) varied depending on the chain lengths of the 3-OH FA. Shorter chain lengths of LPS, known to be produced e.g. by several species of *Pseudomonas* that are ubiquitous in the environment, seemed to be more protective, while longer chain lengths produced by several *Actinobacteria* commonly found in indoor environment were risk factors.

Epidemiological studies may be affected by selection bias and information bias. The schools were arbitrarily selected in a Chinese city, not based on any health complaints. The response rate was high (90.2%), reducing the risk for

Table 8. Kendall tau- β correlations between microbial components and classroom crowdedness and airborne allergens

	MuA	Erg	LPS	C10	C12	C14	C16	C18
Concentration								
Ns/m ²	0.15	0.04	0.24*	0.11	0.15	0.08	0.26*	0.27*
Fel d 1	0.24*	-0.15	0.15	0.16	0.27*	0.11	0.15	0.21
Can f 1	0.23	-0.18	0.08	0.07	0.08	0.06	0.10	0.04
Amount per sample								
Ns/m ²	0.44***	0.45***	0.46***	0.27*	0.44***	0.47***	0.49***	0.55***
Fel d 1	0.40***	0.31**	0.38**	0.21	0.42***	0.43***	0.34**	0.44***
Can f 1	0.23	0.11	0.20	0.19	0.16	0.27	0.16	0.17

Ns/m², no. students per m² of classroom floor; LPS, lipopolysaccharide; MuA, muramic acid; Erg, ergosterol.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

selection bias. Asking pupils of this age about asthma diagnosis or respiratory symptoms would not present any major problem, and identical or similar questions have been used in other Chinese studies in children (29, 30). The high prevalence of respiratory symptoms, particularly daytime attacks of breathlessness, in contrast to the low prevalence of doctor's diagnosed asthma, indicated a possible under-diagnosis of asthma for various reasons (27). This is similar to the pattern of respiratory symptoms in studies in Shanghai, China and South Korea (25, 31). One possible reason for the high prevalence of respiratory symptoms could be the high levels of outdoor air pollution. In a previous study from Hong Kong, ambient SO₂ pollution was shown to be associated with a high prevalence of bronchial hyper-responsiveness in school children (32). However, the strong associations found between reported respiratory symptoms and parental asthma or allergy make the symptom questions more likely to be validly related with asthma, besides indicating the genetic influence (Table 2). One weakness in our study was the lack of clinical diagnosis of asthma because of the limited research resources at the time, but the significant associations in this study nonetheless implied the presence of potentially strong effects.

The health symptoms were collected 1 wk before the environmental measurements, meaning that exposure levels were unknown when the pupils answered the questionnaires, and there were no visible signs of microbial growth in any classroom. Different statistical models were performed, but similar results were obtained and many associations were highly significant ($p < 0.01$). By controlling for current home exposures, we reduced the possibility of confounding because of possible associations between the school environment and the home environment, related with differences in socio-economic status between different areas of the city. In conclusion, we do not believe that our

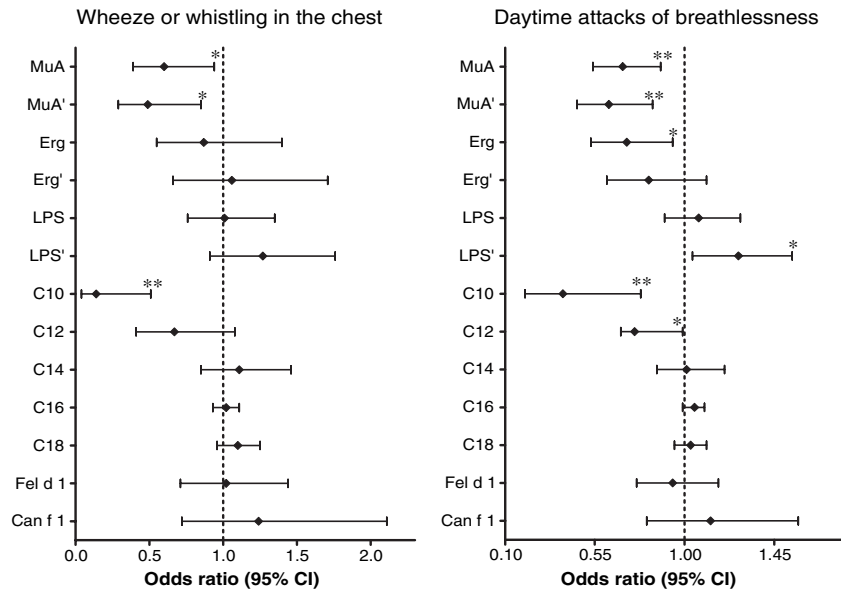
conclusion is seriously affected by selection or information bias, or selection of a particular statistical model. However, the cross-sectional study design limits the possibility to draw conclusions on causal relationships.

In our study, we used tandem-GC-MS, an advanced and highly sensitive and specific chemical-analytical method. It is a measurement with quantification of both culturable and non-culturable microbes. All samples were analysed using internal standards, which ensured good reproducibility and accuracy (22). Three kinds of microbial markers could be measured simultaneously, as well as four different types of LPS. Previously, most studies on microbial exposure focused on one or two markers, usually endotoxin measured by the biological *Limulus* Amebocyte Lysate test (12, 33) or $\beta(1-3)$ glucane from fungi measured by a modified *Limulus* test (34, 35). Very few epidemiological studies have used chemical markers for bacterial exposure, e.g. MuA (18), and only the previous study from Shanghai schools used the same method for all three components (19).

There is little information on microbial exposure in schools, especially bacterial exposure representative by MuA. Most microbial exposure studies have focused on farm or home environment by analysing either endotoxin or fungal exposure or both. As pupils spend most of their daytime in schools, with exposure to different air contaminants, it is an important indoor environment for children. When comparing our data with a previous study in Shanghai, using the same analytical method, the concentrations of MuA and Erg in the settled dust were lower (19). This result was reasonable as Taiyuan, located in the Loess Plateau in the north of China, is much less humid than Shanghai which is in the south of China.

Although in a general lower level of microbial components in the school dust as compared with Shanghai, the variation among the 10 schools

Fig. 1. Graphs for odds ratio (OR) (confidence interval 95%) of associations between concentrations of microbial markers and wheeze and daytime attacks of breathlessness with and without mutual adjustment, as well as for different types of LPS and airborne allergens†. †Controlling for age, gender, parental asthma or allergy, environmental tobacco smoke at home, recent home painting, new floor and new furniture in the past 12 months. MuA', Erg' and LPS' refer to the OR with mutual adjustment.



regarding the microbial level was enough to present the significant associations. The most consistent association was found for MuA, a marker mainly for Gram-positive bacteria, protective for wheeze and daytime attacks of breathlessness, independent of the other two compounds, LPS and ergosterol (Fig. 1). This is in line with the Shanghai school study (19). The associations for wheeze were also consistent with the findings by van Strien on MuA concentrations in mattresses of rural school children using the similar GC-MS method, even after controlling for LPS measurements (18). Our results support the view that possible protective effects of other bacterial components than endotoxin should also be considered. This is also plausible corresponding to its interaction mechanism with TLRs in the innate immunity and regulation roles in human immunological activity (11).

Ergosterol was initially protective for daytime attacks of breathlessness, but not significantly after performing mutual adjustment. Furthermore, it showed positive associations with respiratory infection by mutual adjustment. Most studies on fungal exposure have shown a positive association with respiratory health, including asthmatic symptoms and airway infections (36). Erg in household dust, measured by an old high performance liquid chromatography method, was positively associated with wheeze (37). In addition, the Shanghai school study found a positive association between Erg in classroom dust, measured by tandem GC-MS, and airway infections (19). However, one recent study found that fungal exposure measured as

$\beta(1-3)$ glucans in household dust was protective for atopic wheeze (35). These discrepancies may be explained by the fact that Erg is present both in more toxin moulds and in less harmful yeasts. From a mechanical point of view, the recognition of fungi by the innate immune system appears to be more complex than that of bacteria because fungi can exist in two forms, as hyphae or conidia (4). Therefore, Erg *per se* may not be a health-relevant marker in the general sense and its use should perhaps be limited to strictly defined indoor environments.

Endotoxins played a more complicated role in our study. Shorter chain lengths of 3-OH FA from LPS (C10, C12 and C14) showed protective effects for wheeze or daytime attacks of breathlessness. Longer chain lengths (C14, C16 and C18) tended to be risk factors for asthmatic symptoms. After mutual adjustment, total LPS concentration became a risk factor for daytime attacks of breathlessness. The inconsistency of LPS associations partly reflected the paradoxical nature of endotoxins (12, 38), and different types of LPS could provide new evidence from an epidemiological point of view, for instance, they could reflect different Gram-negative species. Several *Pseudomonas* species that are ubiquitous in the environment produce C10 and C12 3-OH FA, whereas *Actinobacteria* commonly encountered in indoor environments produce C18 3-OH FA; the latter may explain previous findings on poor correlation with the *Limulus* test results (9). To our knowledge, our study is the first epidemiological study investigating the respiratory effects of different types of LPS.

What's more interesting, there were no big changes in the remaining associations for wheeze and daytime attacks of breathlessness, even after controlling for factors of classroom crowdedness and airborne cat allergen levels, and negative associations for MuA and C10 and C12 of LPS were almost the same (data available on request). Thus, the generally unchanged associations were more in favour of the protective effects of microbial components, contrary to the suspicion of the possible effects of selective avoidance because of pet-keeping among pupils (39). This model of regression was performed as a result of the correlations between microbial levels and cat allergens and crowdedness (Table 8), of which the latter two factors could affect pupils' respiratory health (40, 41). This correlation also suggested that the sources of microbial exposure in the settled dust could either be from pupils, together with allergens that came from pet-keeping homes, or from outdoor dust, brought indoors by student activities, as taking off shoes before entering classrooms has never been a habit in Chinese schools. The microbial exposure was more indicative of the general local environmental surroundings of the schools, e.g. the outdoor dust. Further studies are needed to find out the exact sources of microbial exposure. However, the associations for respiratory infection disappeared after controlling for these two new factors. This indicated that other factors related with crowdedness were more liable to predict respiratory infections, independent of microbial exposure.

In addition, one general finding was that the associations were more pronounced if we used amount-per-sample values, as compared with concentration ones. The advantage of measuring amount is that it is not influenced by dilution effects from inert fine dust. We suggest evaluating both amount and concentration in future studies. Moreover, bacterial species related with the different chain lengths of 3-OH FA need to be further investigated.

In conclusion, our study suggested consistently protective effects of microbial exposure indicated by certain chemical markers (MuA and Erg) with respect to respiratory symptoms, but for LPS (endotoxin), the picture is more complex and seems to depend on different types of LPS.

References

1. ASHER M, ANDERSON H, STEWART A, et al. Worldwide variations in the prevalence of asthma symptoms: the International Study of Asthma and Allergies in Childhood (ISAAC). *Eur Respir J* 1998; 12: 315–35.
2. PEARCE N, DOUWES J. The global epidemiology of asthma in children. *Int J Tuberc Lung Dis* 2006; 10: 125–32.
3. VON MUTIUS E, BRAUN-FAHRLANDER C, SCHIERL R, et al. Exposure to endotoxin or other bacterial components might protect against the development of atopy. *Clin Exp Allergy* 2000; 30: 1230–4.
4. VON HERTZEN L, HAAHTELA T. Disconnection of man and the soil: reason for the asthma and atopy epidemic? *J Allergy Clin Immunol* 2006; 117: 334–44.
5. SCHAUB B, LAUENER R, VON MUTIUS E. The many faces of the hygiene hypothesis. *J Allergy Clin Immunol* 2006; 117: 969–77.
6. KEMP A, BJORKSTEN B. Immune deviation and the hygiene hypothesis: a review of the epidemiological evidence. *Pediatr Allergy Immunol* 2003; 14: 74–80.
7. STRACHAN DP. Hay fever, hygiene, and household size. *Br Med J* 1989; 299: 1259–60.
8. SEBASTIAN A, HARLEY W, FOX A, LARSSON L. Evaluation of the methyl ester O-methyl acetate derivative of muramic acid for the determination of peptidoglycan in environmental samples by ion-trap GC-MS-MS. *J Environ Monit* 2004; 6: 300–4.
9. SARAF A, LARSSON L, BURGE H, MILTON D. Quantification of ergosterol and 3-hydroxy fatty acids in settled house dust by gas chromatography-mass spectrometry: comparison with fungal culture and determination of endotoxin by a *Limulus* amoebocyte lysate assay. *Appl Environ Microbiol* 1997; 63: 2554–9.
10. JANEWAY CA, JR. Approaching the asymptote? Evolution and revolution in immunology Cold Spring Harb Symp Quant Biol 1989; 1: 1–13.
11. HEINE H, LIEN E. Toll-like receptors and their function in innate and adaptive immunity. *Int Arch Allergy Immunol* 2003; 130: 180–92.
12. RADON K. The two sides of the 'endotoxin coin'. *Occup Environ Med* 2006; 63: 10.
13. DOUWES J, LE GROS G, GIBSON P, PEARCE N. Can bacterial endotoxin exposure reverse atopy and atopic disease? *J Allergy Clin Immunol* 2004; 114: 1051–4.
14. MICHEL O. Systemic and local airways inflammatory response to endotoxin. *Toxicology* 2000; 152: 25–30.
15. MICHEL O, KIPS J, DUCHATEAU J, et al. Severity of asthma is related to endotoxin in house dust. *Am J Respir Crit Care Med* 1996; 154: 1641–6.
16. NETEA MG, VAN DEUREN M, KULLBERG BJ, CAVAILLON JM, VAN DER MEER JW. Does the shape of lipid A determine the interaction of LPS with Toll-like receptors? *Trends Immunol* 2002; 23: 135–9.
17. SEYDEL U, SCHROMM AB, BLUNCK R, BRANDENBURG K. Chemical structure, molecular conformation, and bioactivity of endotoxins. *Chem Immunol* 2000; 74: 5–24.
18. VAN STRIEN RT, ENGEL R, HOLST O, et al. Microbial exposure of rural school children, as assessed by levels of *N*-acetyl-muramic acid in mattress dust, and its association with respiratory health. *J Allergy Clin Immunol* 2004; 113: 860–7.
19. NORBACK D, MI YH, LARSSON L, WADY L, TAO J, MI YL. Current asthma, respiratory infections and hypersensitivity to moulds in pupils in Shanghai, China, in relation to microbial components in the classrooms. In: *The 9th International Conference on Indoor Air Quality and Climate*. Monterey, California, 2002; 3: 410–5.
20. ASHER MI, MONTEFORT S, BJORKSTEN B, et al. Worldwide time trends in the prevalence of symptoms of

- asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC phases one and three repeat multi-country cross-sectional surveys. *Lancet* 2006; 368: 733–43.
21. MENDELL MJ, HEATH GA. Do indoor pollutants and thermal conditions in schools influence student performance? A critical review of the literature *Indoor Air* 2005; 15: 27–52.
22. SEBASTIAN A, LARSSON L. Characterization of the microbial community in indoor environments: a chemical-analytical approach. *Appl Environ Microbiol* 2003; 69: 3103–9.
23. ASHER MI, KEIL U, ANDERSON HR, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. *Eur Respir J* 1995; 8: 483–91.
24. BURNEY PG, LUCZYNSKA C, CHINN S, JARVIS D. The European Community Respiratory Health Survey. *Eur Respir J* 1994; 7: 954–60.
25. MI YH, NORBACK D, TAO J, MI YL, FERM M. Current asthma and respiratory symptoms among pupils in Shanghai, China: influence of building ventilation, nitrogen dioxide, ozone, and formaldehyde in classrooms. *Indoor Air* 2006; 16: 454–64.
26. SEBASTIAN A, SZPONAR B, LARSSON L. Characterization of the microbial community in indoor environments by chemical marker analysis: an update and critical evaluation. *Indoor Air* 2005; 15 (Suppl. 9): 20–6.
27. ZHAO ZH, ELFMAN L, WANG ZH, ZHANG Z, NORBÄCK D. A comparative study of asthma, pollen, cat and dog allergy among pupils and allergen levels in schools in Taiyuan city, China and Uppsala, Sweden. *Indoor Air* 2006; 16: 404–13.
28. ZHAO ZH. Indoor and outdoor air pollution in relation to allergy and asthma in Taiyuan, China. Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 196. Uppsala: Acta Universitatis Upsaliensis: 70. 2006.
29. CHEN YZ, ZHAO TB, YAN D, et al. A questionnaire-based survey on prevalence of asthma, allergic rhinitis and eczema in five Chinese cities (ISAAC study). *Chinese J Pediatr* 1998; 36: 353–5.
30. CHEN YZ. Comparative analysis of the state of asthma prevalence in children from two nation-wide surveys in 1990 and 2000 year. *Zhonghua Jie He He Hu Xi Za Zhi* 2004; 27: 112–6 (in Chinese).
31. KIM JL, ELFMAN L, NORBACK D. Respiratory symptoms, asthma and allergen levels in schools – comparison between Korea and Sweden. *Indoor Air* 2007; 17: 122–9.
32. WONG CM, LAM TH, PETERS J, et al. Comparison between two districts of the effects of an air pollution intervention on bronchial responsiveness in primary school children in Hong Kong. *J Epidemiol Community Health* 1998; 52: 571–8.
33. WICKENS K, DOUWES J, SIEBERS R, et al. Determinants of endotoxin levels in carpets in New Zealand homes. *Indoor Air* 2003; 13: 128–35.
34. DOUWES J, DOEKES G, MONTIJN R, HEEDERIK D, BRUNEKREEFF B. Measurement of beta(1–>3)-glucans in occupational and home environments with an inhibition enzyme immunoassay. *Appl Environ Microbiol* 1996; 62: 3176–82.
35. SCHRAM-BIJKERK D, DOEKES G, DOUWES J, et al. Bacterial and fungal agents in house dust and wheeze in children: the PARSIFAL study. *Clin Exp Allergy* 2005; 35: 1272–8.
36. BUSH RK, PORTNOY JM, SAXON A, TERR AI, WOOD RA. The medical effects of mold exposure. *J Allergy Clin Immunol* 2006; 117: 326–33.
37. DHARMAGE S, BAILEY M, RAVEN J, et al. Current indoor allergen levels of fungi and cats, but not house dust mites, influence allergy and asthma in adults with high dust mite exposure. *Am J Respir Crit Care Med* 2001; 164: 65–71.
38. LIU AH. Endotoxin exposure in allergy and asthma: reconciling a paradox. *J Allergy Clin Immunol* 2002; 109: 379–92.
39. BORNEHAG CG, SUNDELL J, HAGERHED L, JANSON S. Pet-keeping in early childhood and airway, nose and skin symptoms later in life. *Allergy* 2003; 58: 939–44.
40. TRANTER DC. Indoor allergens in settled school dust: a review of findings and significant factors. *Clin Exp Allergy* 2005; 35: 126–36.
41. WEBER MW, MILLIGAN P, HILTON S, et al. Risk factors for severe respiratory syncytial virus infection leading to hospital admission in children in the western region of the Gambia. *Int J Epidemiol* 1999; 28: 157–62.