



Biomarkers of respiratory allergy in laboratory animal care workers: an observational study

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Abstract

Objectives Laboratory animal allergy is a highly prevalent occupational disease among exposed workers. The aim of the study was to validate the biomarkers of airway inflammation in laboratory animal (LA) care workers.

Methods All of the participants in this observational study (63 LA care workers and 64 controls) were administered a clinical questionnaire, underwent spirometry and a skin prick or radioallergosorbent test for common and occupational aeroallergens, and the fraction of exhaled nitric oxide (FeNO₅₀), exhaled breath condensate hydrogen peroxide (EBC H₂O₂) and serum pneumoprotein levels were measured. Multivariate analysis (ANCOVA) was used to assess the interactions of the variables.

Results FeNO₅₀ levels correlated with exposure ($p=0.002$), sensitisation ($p=0.000$) and age ($p=0.001$), but there was no interaction between exposure and sensitisation when age was considered in the model ($p=0.146$). EBC-H₂O₂ levels were higher in the sensitised workers than in the sensitised controls [0.14 (0.08–0.29) μ M vs 0.07 (0.05–0.12) μ M; $p<0.05$]. Serum surfactant protein A (SP-A) levels were unaffected by exposure, sensitisation or age, although higher levels were observed in symptomatic workers; however, SP-D levels were influenced by exposure ($p=0.024$) and age ($p=0.022$), and club cell 16 levels were influenced by sensitisation ($p=0.027$) and age ($p=0.019$).

Conclusions The presence of the clinical symptoms associated with LA exposure and high FeNO levels should prompt further medical assessments in LA workers. Although EBC-H₂O₂ levels do not seem to reflect eosinophilic inflammation, serum SP-A levels could be used to monitor progression from rhinitis to asthma.

Keywords Laboratory animal allergy · Fraction of exhaled nitric oxide · Hydrogen peroxide · Exhaled breath condensate · Pneumoproteins

Introduction

Laboratory animal allergy (LAA) is considered a significant occupational risk for workers who have regular contacts with laboratory animals (LAs), including scientists, veterinarians,

technicians and cleaners (Bush 2001a, b; Corradi et al. 2011; Wood 2001). The National Institute for Occupational Safety and Health (NIOSH) has reported that there are about two million such workers worldwide [National Institute for Occupational Safety and Health (NIOSH) 1998], up to one-third of whom may develop LAA, and more than 10% of these may develop occupational asthma (Wolfe and Bush 2001).

The most frequent symptoms due to animal allergens (the high-molecular-weight glycoproteins present in animal hair, dandruff, feces, urine and serum) (Corradi et al. 2011) are rhinitis and allergic conjunctivitis (Aoyama et al. 1992; Cullinan et al. 2001), and 4–22% of symptomatic LA workers are affected by asthma (Bush 2001b; Seward 1999).

Primary and secondary prevention measures are essential in order to prevent the onset and progression of LAA. In addition to traditional clinical methods such as anamnesis,

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spirometry and allergy tests, these now include new non-invasive methods for the early detection of biomarkers of susceptibility to lung diseases and their effects (Corradi et al. 2015). For example, the fraction of exhaled nitric oxide (FeNO) is a reliable biomarker of airway inflammation (Adisesh et al. 1998), and hydrogen peroxide (H_2O_2) is considered a valid biomarker of oxidative stress as it is the product of the transformation of the superoxide anion by superoxide dismutase (Adisesh et al. 1998; Chérot-Kornobis et al. 2012; Corradi and Mutti 2005), and high H_2O_2 levels have been documented in patients with lung diseases (Chow et al. 2009; Horváth et al. 1998; Psathakis et al. 2006; Stolarek et al. 2010) and workers exposed to lung irritants (Corradi et al. 2002, 2012; Gube et al. 2010).

The possibility of measuring specific lung-related proteins by means of a moderately invasive procedure such as blood sampling further extends the spectrum of airway-specific biomarkers (Corradi et al. 2015). Surfactant-associated proteins type A and D (SP-A and SP-D) are members of a collagen-like lectin (collectin) family of proteins (Crouch 1998; Fisher et al. 2010; Ledford et al. 2014; Madsen et al. 2003), and both are protective against lung infection as they modulate lung immune responses to viral and bacterial infections (Kurowski et al. 2014). SP-D is also strictly associated with allergic responses as it controls IgE production and histamine release from mast cells (Ledford et al. 2010; Qaseem et al. 2013).

Club cell 16 (CC-16) is secreted by the non-ciliated cells of bronchioles, and has been evaluated as an indicator of airway epithelial barrier disruption (Lakind et al. 2007; Shijubo et al. 2003). Serum CC-16, the levels of which depend on production, leakage through epithelial barrier and renal clearance, has immunomodulatory and anti-inflammatory properties (Broeckaert et al. 2000) as it inhibits the activity of phospholipase A2 and the production of IFN- γ . Moreover, its serum concentrations decrease in the presence of pulmonary diseases, chronic airway inflammation and acute epithelial damage (Corradi et al. 2010).

The aim of this study was to assess the validity of methods of detecting early biomarkers of inflammation and lung damage in LA care workers.

Methods

Study subjects

This observational study involved 63 researchers or technicians involved in LA care, 52 working in university and 11 in pharmacological facilities: 54 (86.8%) were exposed to rats, 46 (73.6%) to mice, 26 (42.1%) to guinea pigs, and 23 (36.8%) rabbits. Concomitant exposure to animals such as pigs, horses, monkeys, hamsters, gerbils, cats and dogs

was rarely reported. The duration of exposure ranged from a minimum of 3 h/month to a maximum of 120 h/month (33.3% were exposed for more than 50 h/month).

Although unmatched, the control group of 64 voluntary university students and employees was not significantly different from the group of LA workers in terms of sex, age, anthropometric data or socio-economic status: 27 had a history of atopy confirmed by a positive skin prick test (SPT) but were not receiving treatment at the time of enrolment and 37 were not atopic.

All of the subjects gave their written informed consent to participate in the study, which was approved by our local Ethics Committee (Comitato Etico Provinciale Parma: Resolution no. 16984/16.05.2013). Their biological samples were handled in accordance with the principles of the Declaration of Helsinki.

Study design

The participants were administered a specific questionnaire covering the characteristics of worktime exposure and respiratory symptoms, and underwent spirometry, FeNO analysis, and blood and exhaled breath condensate (EBC) sampling. A skin prick (SPT) and radioallergosorbent test (RAST) were used to assess atopy. All of the laboratory analyses were carried out at the University of Parma.

Questionnaire

The questionnaire, which was administered by trained medical personnel and completed during the interview, was adapted from Bush (2001b) and designed to collect demographic and clinical data. The participants' occupational histories, working tasks, duration of exposure to LAs, work-related symptoms and their time of onset were carefully recorded. Occupational symptoms were defined as allergy-like symptoms due to causes and conditions attributable to a particular working environment and described in the questionnaire as the temporal relationship between their onset and working activities, and the absence of symptoms when doing different tasks, or during weekends or holidays; work-exacerbated symptoms were defined as pre-existing or concurrent allergy-like symptoms that were worsened by workplace exposure. Domestic exposure to animals was also assessed.

Radioallergosorbent test (RAST)

Serum total immunoglobulin E (tIgE), IgE specific to rat and mouse (serum, epithelia and urine), cat, dog, hamster, guinea pig and rabbit (epithelia), and common Italian aero-allergens (tree, grass, weed and ragweed pollens, house dust and storage mites) were determined in 25 of the LA care workers

using ImmunoCAP (Phadia, Sweden). A specific IgE level of >0.35 KUA/L was considered positive.

Skin prick test (SPT)

All of the participants underwent SPTs using standardised, commercially available allergenic extracts of common aeroallergens (Alk-Abellø A/S, Horsholm, Denmark), including tree, grass, weed and ragweed pollens, house dust mites (*Dermatophagoides farinae* and *D. pteronyssinus*), mould spores (*Alternaria*, *Cladosporium* and *Aspergillus fumigatus*), and storage mites (*Lepidoglyphus destructor*, *Tyrophagus putrescentiae*, *Acarus siro*). Cat, dog, hamster, guinea pig, rat, mouse and rabbit epithelia allergens were also tested (Lofarma, Italy).

SPTs with histamine 10 mg/mL or saline were respectively used as positive and negative controls. The results were read after 15 min, and wheals with a mean diameter of >3 mm were considered positive (Antunes et al. 2009).

Spirometry

Lung function was tested using a portable spirometer (KOKO Spirometer, Sensor Medics, Yorba Linda, CA, USA) in accordance with the current guidelines (Laszlo 2006; Pellegrino et al. 2005) and the 2012 Global Lung Initiative multi-ethnic reference values (Quanjer et al. 2012). The best recordings of forced vital capacity (FVC), forced expiratory volume in 1 s (FEV_1), the FEV_1/FVC ratio and forced expiratory flow at 25–75% of vital capacity ($FEF_{25-75\%}$) were selected chosen.

FeNO

The FeNO was measured in accordance with the American Thoracic Society/European Respiratory Society (ATS/ERS) recommendations (American Thoracic Society, European Respiratory Society 2005), outside pollen seasons and at approximately the same time of day. The measurements were made using a Hypair FENO instrument with the subject in a sitting position: one inhalation of ambient air (NO-free gas was guaranteed by a filter) was followed by exhalation into a mouthpiece at a constant flow rate of 50 mL/s ($FENO_{50}$) for at least 10 s. None of the participants were taking anti-inflammatory drugs and none had had a respiratory tract infection in the previous 2 weeks. All of the subjects were asked to refrain from eating, drinking and smoking during the hour before the measurements were made.

Collection and analysis of EBC biomarkers

EBC was collected using a portable TURBO-DECCS condenser for biomarker research (Medivac, Parma, Italy). The

subjects were asked to breathe tidal volume through the mouthpiece for 15 min when the temperature of the condenser was -5 °C, thus respecting all of the recommended practical standards for EBC collection published by the ATS/ERS (Horváth et al. 2005) as well as anti-contamination principles. The EBC samples (1–2 mL) were shipped in dry ice to the University of Parma laboratory, and then stored at -80 °C. EBC H_2O_2 levels were analysed within 1 month (Corradi et al. 2002) using a commercial Amplex Red Hydrogen Peroxide assay kit (Molecular Probes, Eugene, OR, USA) with a limit of detection (LOD) of 0.01 μ M.

Serum pneumoproteins

Blood was collected during a medical examination, and the serum was stored at -80 °C. The serum concentrations of SP-A, SP-D and CC-16 were measured at the University of Parma laboratory using a commercially available immunoassay kit in accordance with the manufacturer's protocol (BioVendor, Brno, Czech Republic) (Hermans et al. 1998).

Statistical analysis

The sample size was calculated on the basis of the expected FeNO levels and the two factors of exposure (A) and sensitisation (B) (Linhares et al. 2011; Palmberg et al. 2015; Tafuro et al. 2015). Assuming a relative standard deviation within a group of $\sim 33\%$, an effect of A of 20%, and an effect of B of up to 50%, a total of 120 subjects were required in order to provide 30 subjects for each of four cells. This design had 91% power when an F test was used to test factor A at a 5% significance level and the actual standard deviation of the mean values was 10%, and 100% power when an F test was used to test factor B at a 5% significance level and the actual standard deviation of the appropriate mean values was 25%. A lower 10% tolerance was considered acceptable for group composition. We therefore enrolled 127 subjects and divided them into four groups on the basis of exposure (LA care workers vs controls) and sensitisation (sensitised vs non-sensitised).

The normality of the distribution of the quantitative variables was tested using the Kolmogorov–Smirnov test. In the case of a non-normal distribution, the variables were log-transformed and the log-normality was tested. All of the selected biomarkers had a log-normal distribution except for EBC H_2O_2 , for which a substantial number of values were below the LOD. The EBC H_2O_2 data were therefore expressed as median values (interquartile range) and the values below the LOD were assigned the value of half the LOD. Crude differences between groups in the log-normal variables were tested by means of an independent sample t test or analysis of variance (ANOVA) followed by Tukey's post hoc test, and those

in EBC H₂O₂ were tested by means of the Mann–Whitney/Kruskal–Wallis test followed by Dunn's post hoc test. Simple correlations were made using parametric (Pearson) or non-parametric (Spearman) tests depending on the normality of the residuals. Fisher's exact test was used for the qualitative variables (because of the sample size) and the odds ratio (OR) was calculated. When multiple fixed factors/covariates showed significant crude effects, two-way ANOVA was used to test the main effects of two categorical predictors and their interactions, with multiple regression/analysis of covariance (ANCOVA) models being used in the presence of quantitative predictors. In the case of at least two factors and at least two covariates, the multiple regression model was preferred in order to make the results clearer, although the *p* value of main effects is the same in both multiple regression and ANCOVA models. The most frequently used categorical predictors were workers/controls and sensitised/non-sensitised, and age was almost always used as a continuous covariate, although predicted FEV₁% was also included in the case of CC-16.

The data were analysed using IBM SPSS v. 22 (IBM, Armonk, NY, USA) and Graphpad Prism v. 5.0 software (Graphpad, La Jolla, CA, USA), with *p* = 0.05 being set as the level of significance.

Results

Table 1 shows the main characteristics of the subjects. There was no statistical difference in the age or lung function parameters of the LA workers and controls.

Questionnaire

Thirty-seven LA care workers (59%) reported allergy-like symptoms: 18 rhino-conjunctivitis alone, 15 rhino-conjunctivitis and asthma-like symptoms, two eczema, and two cutaneous, rhino-conjunctivitis and asthma-like manifestations. Analysis of the relationship between symptom onset and working activity showed that 18 of the 37 symptomatic workers had symptoms that were not specifically work related, and 19 (30% of all LA care workers) had work-related symptoms (eight with occupational symptoms and 11 with work-exacerbated symptoms).

Allergological tests (SPT and RAST)

Table 2 shows the SPT and RAST results. Eleven of the 18 workers with work-related symptoms had a positive allergological test relating to the LAs they were exposed to (three a positive RAST and nine a positive SPT) and seven an allergy to storage mites (all of whom were co-sensitised

Table 1 Main study subjects' characteristics

	LA workers	Controls
Number (M/F)	63 (27/36)	64 (30/34)
Age median (min–max)	35 (24–70)	31 (21–58)
Smoking (not/ex/current)	47/8/8	33/12/19
Smoker number of pack/year median (IQR 25–75 pct)	3.75 (1–16)	11.5 (6.2–23.1)
Exposure period, months median (IQR 25–75 pct)	84 (48–240)	NA
Pet owner (yes/no)	28/35 (44/56%)	30/34 (47/53%)
Allergy-like symptoms	37 (59%)	33 (51.6%)
FEV ₁ % pred. median (IQR 25–75 pct)	101.7 (93.7–110.5)	100.2 (94.2–108.0)
FVC % pred median (IQR 25–75 pct)	104.8 (96.4–111.0)	102.7 (93.9–107.9)
FEV ₁ /FVC median (IQR 25–75 pct)	0.81 (0.77–0.84)	0.80 (0.76–0.84)

IQR interquartile range, *pct* percentile, *FEV₁* forced expiratory volume in the 1st second, *FVC* forced vital capacity, *Pred* predicted values

Table 2 Results of positive allergological tests

	Pollen, <i>N</i> (%)	DM, <i>N</i> (%)	SM, <i>N</i> (%)	Moulds, <i>N</i> (%)	Domestic animal, <i>N</i> (%)	LA, <i>N</i> (%)
LA care workers (33)	22 (66%)	20 (61%)	9 (27%)	3 (9%)	20 (61%)	16 (48%)
Controls (27)	20 (74%)	14 (52%)	7 (26%)	4 (15%)	8 (30%)	1 (4%)

LA laboratory animals (considered positive only if those workers with a positive allergological test to LA at work), *SM* storage mites, *DM* dust mites, *N* numbers

to house dust mites). Among the 26 asymptomatic workers, only two were sensitised to an LA (rats) (Table 3).

On the basis of symptomatic sensitisation to LA epithelia, the prevalence of work-related allergic diseases was 17.5% (11/63), but this increased to 25.4% (16/63) when including those who were asymptotically sensitised to LA epithelia, and to 39.7% (25/63) when including those who were sensitised to storage mites (25/63). The prevalence of LA sensitisation was higher among the workers than the controls [16/63 vs 1/64; OR 21.44 (3.06–913)].

Ten of the symptomatic workers sensitised to LAs reported asthma-like symptoms and rhino-conjunctivitis, and two reported rhino-conjunctivitis alone; none reported eczema.

Twenty-six of the 63 LA care workers and 26 of the 64 controls were pet owners, and pet sensitisation was recorded in respectively 20 and eight members of the two groups (Table 2), a difference that was statistically significant ($p=0.004$, Fisher's exact test). However, only five of 20 LA care workers sensitised to domestic animals actually owned pets, thus indicating that sensitisation was less frequent among the pet-owning workers ($p=0.034$, Fisher's exact test). Seven of the eight pet-sensitised controls had pets as against only five of the 20 pet-sensitised LA care workers (Table 4). Owning a pet was therefore considered a possible confounding factor for all of the measured biomarkers (t test of log values), but no statistical difference was found.

Spirometry

There were no significant differences in spirometric values between the LA care workers and controls (Table 1). On the basis of the ATS/ERS interpretative criteria, none of the workers had obstructive abnormalities, and only one had a restrictive pattern ($FEV_1/FVC > LLN$ and $FEV_1 < LLN$). One of the controls had a mixed pattern.

Table 4 Sensitisation and pet ownership in LA care workers and controls

	LA care workers (pet sensitised) 20/33 (61%)	Controls (pet sensitised) 8/27 (30%)
Pet owner (<i>N</i>)	5	7
No pets (<i>N</i>)	15	1

N numbers

FeNO

Table 5 and Fig. 1 show FeNO values in the LA care workers and controls (divided on the basis of sensitisation to at least one aero-allergen), which were analysed in order to investigate the effects of sex, smoking history (pack/years), age, body mass index (BMI), lung function parameters, sensitisation and exposure.

FeNO values were not affected by sex, BMI or lung function parameters, but they were influenced by exposure ($p=0.002$), sensitisation ($p=0.000$) and age ($p=0.001$). No interaction between exposure and sensitisation was demonstrated when considering age ($p=0.146$, ANCOVA) (Table 6). Smoking history did not have any effect (ANCOVA model), although there was a statistically significant difference in FeNO levels between non-smoking sensitised LA care workers and controls [geometric mean values and 95% confidence intervals: 37.76 (26.76–53.27) ppb vs 18.79 (15.25–23.12) ppb; $p=0.035$].

FeNO levels were > 50 ppb (a cut-off value for eosinophilic airway inflammation) in 18 LA care workers (Dweik et al. 2011), and significantly correlated with H_2O_2 levels (Spearman's ρ 0.2, $p=0.024$) and SP-D concentrations (Spearman's ρ 0.22, $p=0.013$).

Table 3 Relation between allergological results and symptoms in LA care workers

	LA, <i>N</i> (%)	PETs, <i>N</i> (%)	SM, <i>N</i> (%)
Occupational symptoms ^a (8)	4 (50%)	4 (50%)	3 (37%)
Work-exacerbated symptoms ^b (11)	7 (63%)	8 (72%)	4 (36%)
Symptoms not related to work exposure ^c (18)	3 (16%)	6 (33%)	2 (11%)
Asymptomatic (26)	2 (7%)	2 (7%)	0 (0%)

LA laboratory animals, SM storage mites

^aOccupational symptoms: allergy-like symptoms due to causes and conditions attributable to a particular work environment

^bWork-exacerbated symptoms: pre-existing or concurrent allergy-like symptoms that are worsened by workplace exposures

^cSymptoms not related to work exposure: symptoms present independently from workplace exposures

Table 5 Biomarkers in LA care workers and controls

	LA care workers		Controls	
	Sensitised <i>N</i> 33 (52.4%)	Non-sensitised <i>N</i> 30 (47.6%)	Sensitised <i>N</i> 27 (42.2%)	Non-sensitised <i>N</i> 37 (57.8%)
FeNO ₅₀ (ppb) geometric mean [GSD]	36.18 [2.38]	19.34 [1.87]	18.25 [1.63]	16.32 [1.66]
H ₂ O ₂ (uM) median [IQR]	0.14 [0.08–0.29]	0.09 [0.04–0.27]	0.07 [0.05–0.12]	0.11 [0.04–0.22]
SP-A (ng/mL) geometric mean [GSD] serum physiological range 20–200 ng/mL	72.97 [3.71]	43.76 [2.28]	42.01 [$\pm$ 2.18]	40.99 [1.73]
SP-D (ng/mL) geometric mean [GSD] serum physiological range up to 60 ng/mL	56.34 [1.75]	51.60 [1.58]	39.76 [1.96]	40.32 [2.21]
CC-16 (ng/mL) geometric mean [GSD] serum physiological range 5–50 ng/mL	6.93 [1.81]	8.60 [1.53]	5.80 [2.10]	9.19 [1.87]

We defined as “sensitised” those subjects with at least one positive allergen (SPT or RAST)

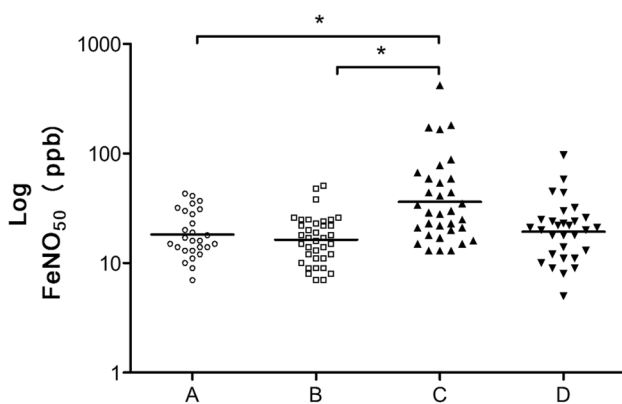


Fig. 1 FeNO values (geometric mean). *A* sensitised control, *B* non-sensitised control, *C* sensitised LA workers, *D* non-sensitised LA workers. * $p < 0.05$

H₂O₂ in exhaled breath condensate

Table 5 and Fig. 2 show EBC H₂O₂ levels in the LA care workers and controls, divided on the basis of allergic

sensitisation. They were not statistically different between LA care workers and controls as a whole ($p = 0.085$), but the sensitised LA care workers had significantly higher levels than the sensitised controls ($p < 0.05$).

Serum pneumoproteins

Table 5 shows serum pneumoprotein levels in the LA care workers and controls, divided on the basis of allergic sensitisation, and Table 7 the results of the multiple regression analysis.

Serum SP-A levels were not affected by LA exposure, sensitisation or age. Among the workers with LAA, the presence of symptoms (rhino-conjunctivitis) was associated with higher levels of SP-A in the univariate analysis ($p < 0.01$) and in the multivariate analysis after correcting for sensitisation and the presence of asthma ($p < 0.05$) (not shown). SP-A levels were above the physiological range in six LA care workers and one control.

Table 6 Results of ANCOVA test for FeNO₅₀ as dependent variable

Source	Type III sum of squares	<i>df</i>	Mean square	<i>F</i>	<i>p</i>
Corrected model	3.313	4	0.828	11.638	0.000
Intercept	11.000	1	11.000	154.547	0.000
Age	0.898	1	0.898	12.620	0.001
Exposure	0.749	1	0.749	10.524	0.002
Sensitisation	1.356	1	1.356	19.051	0.000
Exposure, sensitisation	0.153	1	0.153	2.144	0.146
Error	8.683	122	0.071		
Total	236.760	127			
Corrected total	11.997	126			

$R^2 = 0.276$ (adjusted $R^2 = 0.252$)

F = slope; in bold significant values

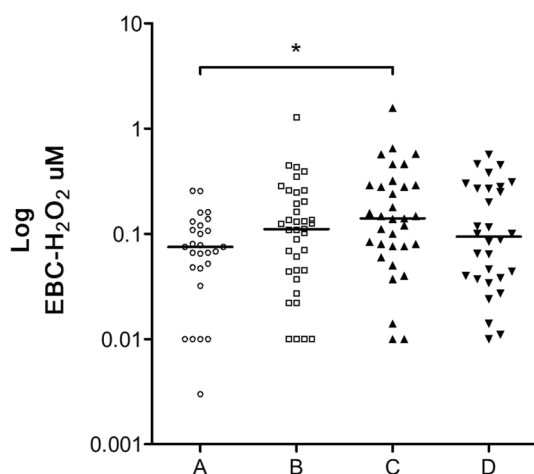


Fig. 2 EBC- H_2O_2 values (median). *A* sensitised control, *B* non-sensitised control, *C* sensitised LA workers, *D* non-sensitised LA workers. $*p < 0.05$

Table 7 Results of multiple regression tests for log SP-A, log SP-D and log CC-16 and different predictors

	Unstandardised coefficients		<i>p</i>
	<i>B</i>	Std. error	
Dependent variable log SP-A			
Exposure	0.126	0.072	0.083
Sensitisation	0.122	0.076	0.108
Age	0.001	0.003	0.775
Dependent variable log SP-D			
Exposure	0.112	0.049	0.024
Sensitisation	0.061	0.052	0.236
Age	0.005	0.002	0.022
Dependent variable log CC-16			
Exposure	−0.001	0.045	0.989
Sensitisation	−0.107	0.047	0.027
Age	0.005	0.002	0.019
Sex	−0.088	0.045	0.052
FEV ₁ % pred	0.003	0.002	0.081

B slope; in bold significant values

Occupational exposure ($p = 0.024$) and age ($p = 0.022$) increased serum SP-D levels, which were higher than the normal upper limit in 26 LA care workers and 20 controls.

As serum CC-16 levels significantly correlated with predicted FEV₁% (Pearson $r = 0.2$; $p = 0.023$), FEV₁ was added to the ANCOVA model. Exposure had no effect, but sensitisation and age respectively had a negative and positive effect. CC-16 levels were lower than the physiological range in eight LA care workers and 16 controls.

Serum pneumoprotein levels did not correlate with each others, but serum SP-D levels correlated with FeNO₅₀ levels

(Spearman's $r = 0.22$, $p = 0.01$), and serum SP-A levels with H₂O₂ levels (Spearman's $r = 0.22$, $p = 0.01$).

Discussion

The large proportion of LA care workers reporting allergic symptoms found in this study (37/63, 59%) is in line with the 40% found in a large-scale study of Western Europeans (Bauchau and Durham 2004), but the percentage of workers exposed to LA for more than 50 h/month (33.3%) is higher than that indicated in other published studies (Greiner et al. 2011; LareseFilon et al. 2002). It is known that LA care workers are at high risk of sensitisation, and our finding of a 17.5% prevalence of work-related allergy in LA-sensitised workers (11/63) is within the range of 6–44% reported studies published between 1980 and 2015 (Corradi et al. 2011; Palmberg et al. 2015; Ruoppi et al. 2005). The most frequently reported symptoms were rhino-conjunctivitis and asthma: 12/63 (19%) reported occupational rhino-conjunctivitis alone and 10/63 (15.9%) also complained of asthma, once again in line with previous findings (LareseFilon et al. 2002; Pacheco et al. 2003; Suarathana et al. 2009). However, it is not easy to distinguish irritation from allergic responses and there are many cleaning and other chemicals that may affect work-related symptoms; furthermore, late allergic responses should also be considered when investigating causal relationships between symptoms and working exposure.

Sensitisation or allergies to cats and dogs have traditionally been considered independent risk factor for the development of LAAs (Gautrin et al. 2001; Hollander et al. 1996; Krakowiak et al. 2007; Suarathana et al. 2009). Our findings indicate that owning a pet did not interfere with LA sensitisation, and that LA care workers without pets also show evidence of sensitisation to cats or dogs. This may be due to cross-reactions to antigenic proteins, such as lipocalins and albumins, as published data suggest that cross-reacting mechanisms play a role in a significant proportion of allergic sensitisations to furry pets and unusual/exotic mammals even in the absence of any direct or indirect contacts (Konradsen et al. 2015; Liccardi et al. 2015).

The spirometry data did not indicate any obstruction in the LA care workers, but asthma-like symptoms were reported. However, obstructive abnormalities can be difficult to detect during occupational screening because asthma-related obstruction may vary over time.

We explored the possible effects of sex, age, smoking history, sensitisation and exposure on FeNO levels in all of the subjects. Smoking history did not seem to have any effect, probably because of the small number of smokers ($n = 27$) and pack/years, but FeNO levels were positively influenced by the presence of sensitisation (positive allergological

tests). It has long been debated whether FeNO can identify atopy or airway inflammation (Olin et al. 2004), but it is generally accepted that it is a marker of airway inflammation because it reflects the eosinophilic inflammatory process observed in the airways of asthmatic patients (Alving et al. 1993; Ricciardolo et al. 2004; Suresh et al. 2007).

H₂O₂-EBC concentrations were not significantly higher among the LA workers than among the controls, which is in line with the view that H₂O₂ seems to be more related to a neutrophilic response than eosinophilic inflammation (Fireman et al. 2008). None of the subjects had any symptoms of acute inflammation at the time of enrolment, but H₂O₂ levels were significantly higher in the sensitised LA care workers than in the sensitised controls ($p < 0.05$) because of the effect of occupational exposure to allergens that can trigger oxidative stress.

SP-A values were not influenced by LA exposure, sensitisation or age, which is in line with reports that serum SP-A levels are not statistically different in healthy smokers, asthmatics and patients suffering from COPD (Mutti et al. 2006), although it has been found that surfactant protein levels may be either increased or decreased in people with allergic diseases (Erpenbeck et al. 2009; Ledford et al. 2014). Above normal SP-A levels were recorded in 28.1% of our sensitised LA care workers, and the same workers reported rhino-conjunctivitis. This is in line with published data showing that SP-A levels depend on the severity of allergic rhinitis (Ledford et al. 2014).

The increase in serum SP-D levels in the LA care workers may have been due to alterations in the permeability of the alveolar-capillary membrane probably induced by innate immunity mechanisms as a result of the SP-D-mediated opsonisation of allergens (Qaseem et al. 2013). SP-D levels were not influenced by atopic status or smoking habits and so, on the basis of our findings, SP-D may be considered a marker of current allergen exposure.

CC-16 values were influenced by age and sensitisation, but exposure had no effect. The effect of age is in line with previous observations, and it has been postulated that age-related changes are secondary to increased alveolar capillary leakage (Carbonnelle et al. 2002; Hermans et al. 2003). Our observation of an association between CC-16 values and predicted FEV₁% is in line with the demonstration of a correlation between lung function (FEV₁/FVC) and serum CC-16 levels in asthmatic patients, (Ye et al. 2004). Intermittent allergic rhinitis is associated with decreased CC-16 levels in nasal lavage fluids (Benson et al. 2007; Johansson et al. 2005), and it has been hypothesised that decreased CC-16 expression may be related to the pathogenesis of the condition. Our findings seem to be in line with the view of decreased CC-16 levels at the time of exposure.

The main limitation of this study is that we did not use a methacholine test or more invasive tests such as a sputum

cell count or bronchoalveolar lavage to demonstrate airway inflammation and correlate it with our biomarkers. However, the prevalence of LAA in our study was immunologically proven even if we did not use the same test (SPT/RAST).

In conclusion, high bronchial FeNO values should prompt occupational health practitioners to encourage workers to undergo an SPT or methacholine test, especially if they are symptomatic. Although EBC H₂O₂ levels do not seem to be a useful means of revealing eosinophilic inflammation, serum SP-A levels may be used to monitor progression from rhinitis to asthma. Our data demonstrate that conventional health surveillance programmes that are mainly based on spirometry are insufficient to identify populations at risk of LA sensitisation. LA care workers should be informed of the occupational risk of LA sensitisation and periodically evaluated using breath and blood biomarkers whose changes can precede the clinical phase of LAA.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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