Clin Physiol Funct Imaging (2015) 37, pp489–497 doi: 10.1111/cpf.12323

Exhaled particles as markers of small airway inflammation in subjects with asthma

Per Larsson[1], Mona Lärstad[1], Björn Bake[2], Oscar Hammar[1], Anna Bredberg[1], Ann-Charlotte Almstrand[1], Ekaterina Mirgorodskaya[1] and Anna-Carin Olin[1]

[1]Occupational and Environmental Medicine, Department of Public Health and Community Medicine, Institute of medicine, Sahlgrenska Academy, University of Gothenburg, and [2]Respiratory Medicine and Allergy, Department of Internal Medicine, Institute of medicine, Sahlgrenska Academy, University of Gothenburg, Sweden

Correspondence
Per Larsson, Sahlgrenska Academy, University of Gothenburg, Box 414, 405 30 Göteborg, Sweden
E-mail: per.larsson@amm.gu.se

Accepted for publication
Received 21 May 2015;
accepted 22 October 2015

Key words
aerosol; airway opening; albumin; asthma; birch pollen; exhaled breath condensate; exhaled particles; inflammation; surfactant protein A

Summary

Exhaled breath contains suspended particles of respiratory tract lining fluid from the small airways. The particles are formed when closed airways open during inhalation. We have developed a method called Particles in Exhaled air (PExA®) to measure and sample these particles in the exhaled aerosol. Here, we use the PExA® method to study the effects of birch pollen exposure on the small airways of individuals with asthma and birch pollen allergy. We hypothesized that birch pollen-induced inflammation could change the concentrations of surfactant protein A and albumin in the respiratory tract lining fluid of the small airways and influence the amount of exhaled particles. The amount of exhaled particles was reduced after birch pollen exposure in subjects with asthma and birch pollen allergy, but no significant effect on the concentrations of surfactant protein A and albumin in exhaled particles was found. The reduction in the number of exhaled particles may be due to inflammation in the small airways, which would reduce their diameter and potentially reduce the number of small airways that open and close during inhalation and exhalation.

Introduction

The small airways of the lungs are rather inaccessible, which makes it difficult to study their responses to environmental exposure. To obtain samples from the small airways in a non-invasive way, our department has developed a method called Particles in Exhaled air (PExA®) (Almstrand *et al.*, 2009). An aerosol containing liquid particles of respiratory tract lining fluid (RTLF) is formed in the small airways when performing a breathing manoeuvre that induces airway closure and re-opening (Johnson & Morawska, 2009; Almstrand *et al.*, 2010; Haslbeck *et al.*, 2010; Schwarz *et al.*, 2010). The PExA® instrument (patent P17584SE00) counts and samples these particles, providing RTLF samples of amounts in the mid-nanogram range that can be characterized using various analytical techniques. Inflammation in the small airways can increase the permeability of the blood–air space barrier, leading to transudation of blood plasma into the airway space and leakage of lung proteins out from the airways. This in turn may change the protein content and depth of the RTLF (Widdicombe, 2002), leading to changes in its physical properties that could influence the processes of airway opening and closure as well as the quantity and composition of exhaled RTLF-derived aerosol particles.

Surfactant protein A (SP-A) has a number of functions that make it interesting as a potential biomarker for inflammation in the lower airways. SP-A is mainly produced in the alveoli and is considered to have an important role for surfactant homoeostasis in the alveolar compartment, but participation in the innate immune system host defence is the major function of SP-A. SP-A binds to a variety of microorganisms and acts as an opsonin that promotes their clearance and may also act as modulator of the immune response. (Hermans & Bernard, 1999; Wright, 2005; Kishore *et al.*, 2006; Perez-Gil, 2008) Inflammation in general is associated with a leakage of albumin out from the intravascular compartment to surrounding extracellular compartments (Nicholson *et al.*, 2000). If there is a large concentration gradient between the vascular compartment and the alveolar compartment then increased albumin concentration in RTLF might be associated to small airways inflammation.

This work explores the effects of seasonal birch pollen exposure on exhaled particles in subjects with asthma and birch pollen allergy. The aims were to determine the total mass of exhaled particles in the size range 0·4–4·6 μm in diameter and the concentrations of SP-A and albumin in exhaled particles during and outside pollen season as markers of small airways inflammation.

Methods

Ethics and participants

Thirteen non-smoking subjects (five females and eight males) between the ages of 22 and 61 diagnosed with mild intermittent asthma were recruited. All subjects were determined to be allergic to birch pollen based on the presence of IgE antibodies against birch pollen in their blood (Phadiatop; Pharmacia & Upjohn Diagnostics, Uppsala, Sweden) and self-reported allergy symptoms. Several subjects with asthma were also positive to one of the other allergens in the Phadiatop test such as grass, mite and dog/cat/horse. They were clinically stable and had either no or mild symptoms outside of pollen season. Four of the subjects were taking long-acting β2 stimulators, six were regularly using inhaled glucocorticoids and one subject was using oral corticosteroid and corticosteroid injections. The remaining subjects were only using bronchodilators on demand. The long-acting β2 stimulators were withheld for 2 days before the clinical examination. Thirteen healthy, non-smoking subjects aged between 17 and 63 (five females and eight males) were recruited as a control group. All control subjects tested negative for the presence of IgE antibodies against birch pollen and the other antigens included in the Phadiatop test had no history of lung disease and did not use asthma medication. None of the subjects had suffered from any respiratory tract infections during the 3 weeks immediately prior to the clinical tests. The subjects were examined during the pollen season of 2011 (i.e. between the end of April and the beginning of June), and outside the pollen season during the autumn of either 2010 or 2011. Birch pollen concentrations were measured using Burkard Seven Day Volumetric Spore Trap, and the results were reported by the Botanical Analysis Group (Botanical Analysis Group AB, Gothenburg, Sweden).

All subjects gave their written informed consent, and the study protocol was approved by the Human Research Ethics Committee of the Medical Faculty at the University of Gothenburg, Sweden.

Study design

The study involved two series of lung function tests and measurements of exhaled particles, one conducted outside pollen season and one during pollen season. For each participant, particles were measured and sampled before and after bronchodilatation. SP-A and albumin concentration in particles as well as the amount of particles per litre exhaled breath before and after birch pollen exposure were determined. Exhaled nitric oxide (measured as fractional exhaled nitric oxide, FENO) was used as an inflammation marker to link changes in exhaled particles to underlying inflammation.

A control group was included in the study to control for effects of seasonal change that were unrelated to asthma and pollen allergy.

Spirometry

Spirometry (Spirare SPS310 sensor and Spirare 3 software; Diagnostica AS, Oslo, Norway) was performed before and after bronchodilatation (3*0·5 mg Bricanyl; AstraZeneca; Sweden) and in accordance with the ATS/ERS criteria (Miller *et al.*, 2005). The forced vital capacity (FVC) and the forced expired volume in the first second (FEV_1) were measured, and the ratio FEV_1/FVC was calculated and expressed as a percentage. Per cent predicted values were generated using the ECCS/ERS reference equations (Quanjer *et al.*, 1993).

Particle measurement and sampling

The design of the PExA® instrument and the set-up for measurement of exhaled particles have been previously described in detail by Almstrand *et al.* (2009). Particles in exhaled air with an aerodynamic diameter of 0·4–4·6 μm that can be measured and sampled by the PExA® instrument are in this study referred to as PEx. To increase the amount of collected material, a breathing manoeuvre that increases particle formation was used during sampling of exhaled breath (Johnson & Morawska, 2009; Almstrand *et al.*, 2010). All subjects were instructed to perform a maximal exhalation to the lung residual volume, hold their breath for 3-s and then perform a maximal inspiration followed by a relaxed exhalation into the PExA® instrument. Test subjects inhaled particle-free air through a HEPA filter and exhaled into the PExA® instrument via a two-way valve that could direct the flow into the instrument or to ambient air. The mass median aerodynamic diameter of exhaled particles during sampling is between 0·7 and 1·5 μm (unpublished data) from P. Larsson and A-C. Olin while the particle number concentrations have a broad maximum between 0·4 and 0·6 μm (Holmgren *et al.*, 2010; Schwarz *et al.*, 2015). An ultrasonic flow meter (OEM flow sensor, Spiroson-AS, Medical Technologies, Zürich, Switzerland) measures exhaled flow and the exhaled volume. In the PExA® instrument, exhaled particles are drawn through an inertial impactor (PM10 Impactor; Dekati Ltd., Tampere, Finland) using a vacuum pump. The impactor was set up to sample particles with an aerodynamic diameter of 0·5–7·0 μm. Particles are sampled by impaction on a hydrophilic silicon wafer inside the impactor. The number of particles sampled on the silicon wafer is estimated based on measurements from an optical particle counter (Grimm model 1·108; Grimm Aerosol, Ainring, Germany), which is used to measure particle sizes and particle number concentrations in a fraction of the air that is drawn through the impactor. To more accurately represent the sizes of exhaled particles, the particle size intervals reported by the particle counter were adjusted upwards from 0·3–3·0 to 0·4–4·6 μm as described by Holmgren *et al.* (2010). The characteristic particle volume in each size interval was estimated using the expression for the volume of a sphere. Endogenous particles formed from RTLF in small airways are expected to contain water, lipids, proteins and less

than one per cent of salts (Knowles *et al.*, 1997; Bredberg *et al.*, 2012). Their density is thus expected to be similar to that of water, that is 1000 kg m^{-3} (Schurch *et al.*, 1992; Bernhard *et al.*, 1997). The particle mass sampled on the silicon wafer in the impactor is calculated from the particle concentrations measured by the particle counter, the total volume sampled by the impactor and the expected density of the particles, that is estimated particle mass sampled = impactor sampling efficiency × impactor operating flow rate × sampling time × average particle number concentration × particle volume × particle density.

Chemical analysis

Extractions and quantifications of SP-A and albumin were carried out on pooled PEx samples generated by combining 60 l exhalations from the same participant before and after bronchodilatation to maximize the amount of sampled proteins. Particles deposited on the silicon wafer were stored dry in Teflon containers for 6–30 months at −80°C. An extraction buffer was prepared from phosphate-buffered saline (PBS) (10 mM PBS, 0·15 M NaCl, pH 7·4), containing 0·05% TWEEN®20 (v/v) and 1% BSA (w/v) (Prod #: 28352, 37525; Thermo Scientific, Rockford, IL, USA). The silicon support containing the sample was placed with the sample surface facing down in a glass vial, followed by 100 μl of extraction buffer and 225 μl of Milli-Q® water (Millipore Synergy; Millipore, Billerica, MA, USA). Extraction conditions were preheating to 37°C for 10 min in a water bath followed by 2 min ultrasonication and 60 min of shaking at 400 RPM at room temperature (21°C). After extraction, the sample volume was split between SP-A and albumin analysis. The samples were quantified by enzyme-linked immunosorbent assay (ELISA). On the day of quantification, each sample was made up to the required volume for the assays by dilution with assay diluent supplied by the manufacturer (100 μL diluent to 125 μL of extracted sample). Standards and controls were prepared with a diluent whose composition was chosen to match the sample matrix, using a 100:125 ratio of assay diluent to extraction solvent. SP-A ELISA (BioVendor, Brno, Czech Republic) and albumin ELISA (E-80AL; Immunology Consultant Laboratory, Newberg, OR, USA) were performed according to the manufacturer's instructions with the exception of the modified dilution buffer used with the control and standard samples. All samples were run in duplicate; the average coefficient of variation for duplicates was 5·7% for the SP-A ELISA (95% confidence interval 4·6–6·9%, median concentration 8 ng ml^{-1}) and 4·4% for the albumin ELISA (95% confidence interval 3·6–6·2%, median concentration 16 ng ml^{-1}). On the ELISA plates, paired samples were placed next to one another to minimize variability within individuals while samples from subjects with asthma and control subjects were alternated to avoid systematic differences between groups.

The concentrations of albumin and SP-A in PEx are expressed as weight per cent (wt%) and obtained by dividing the masses of SP-A and albumin extracted from the silicon wafer by the mass of PEx collected on the wafer.

To estimate the variation between ELISA runs, a pooled PEx sample was prepared. The pooled sample was extracted and split such that duplicate subsamples could be analysed in each of the three SP-A and two albumin runs. Assays of the pooled PEx control samples yielded SP-A concentrations of 11·3, 11·1 and 11·2 ng ml^{-1}, and albumin concentrations of 67·4 and 60·8 ng ml^{-1}. Preliminary investigations demonstrated that the assay exhibited good repeatability with acceptable performance in spiking/recovery experiments and responded linearly to increasing sample dilution (data not presented). The storage stability and extraction recovery of samples similar to those used in this work has been tested previously: SP-A was found to be stable for at least 8 months, and its recovery from spiked silicon wafers was 93% (Larsson *et al.*, 2012).

Exhaled nitric oxide

The protocol used to measure FENO levels and the effects of pollen exposure on FENO are described elsewhere (Bake *et al.*, 2014). FENO measurements were performed using the NIOX system (NIOX; Aerocrine AB, Stockholm, Sweden) on the same day as the PEx sampling. The measurements were performed in duplicate prior to spirometry, at three target flow rates: 0·05, 0·10 and 0·27 l s^{-1} (the corresponding FENO measurements are referred to as $FENO_{0·05}$, $FENO_{0·10}$ and $FENO_{0·27}$, respectively). In all cases, the achieved flow rate was within 10% of the target value.

Statistical analysis

Differences between seasons for paired observations were tested using the Wilcoxon signed rank-sum test and comparisons between independent groups were tested using Wilcoxon–Mann–Whitney U test. For the Wilcoxon signed rank-sum tests relating to differences between seasons, the data for each observation were log-transformed in cases where this improved the symmetry of the data around the median inter-seasonal difference. Spearman correlation (r_s) was used to assess the correlations between the PEx variables (SP-A wt%, albumin wt%, PEx ng l^{-1}) and FENO. Statistical tests were two-sided, and P-values of $<0·05$ are referred to as significant. All statistical analyses were performed using SAS (Statistical Analysis System, version 9.3; SAS Institute Inc., Cary, NC, USA).

Results

Demographics

General information on the subjects is presented in Table 1, which shows that the subjects with asthma and the controls were broadly similar but the subjects with asthma had a higher average weight.

Table 1 Physical characteristics of the test subjects.

	Subjects with asthma (n = 13)	Control subjects (n = 13)
Age (years)	41 (35, 49)	46 (41, 59)
Height (cm)	173 (169, 181)	169 (165, 175)
Weight (kg)	90 (76, 93)	72 (69, 76)

Median values are presented, with first and third quartiles in parentheses.

Birch pollen levels

The pollen season of 2011 was characterized by a peak count of 2800 grains m^{-3} at the end of April, with elevated counts persisting until the second week of May. Figure 1 shows the pollen counts measured during the pollen season of 2011.

Spirometry and $FENO_{0\cdot05}$

Spirometry values were normal during and outside the pollen season, and the results for the subjects with asthma were similar to those for the controls (Table 2). There were no significant changes in spirometry between seasons in either group. The effect of bronchodilation on FEV_1 was small but significantly larger in the subjects with asthma than in the controls, and there were no significant differences between seasons for either subjects with asthma or control subjects. $FENO_{0\cdot05}$, however, showed substantial effect of season among subjects with asthma.

Exhaled particles

The total mass of exhaled particles were significantly lower in the samples collected from subjects with asthma during pollen season compared to outside of pollen season, as shown in Tables 3 and 4, as well as the individual results presented in Fig. 2. The particle mass per litre of exhaled breath did not change significantly after bronchodilatation among either the subjects with asthma or the control subjects (data not presented). Furthermore, there were no significant differences between subjects with asthma using inhaled glucocorticoids and those not using inhaled glucocorticoids. The results from the subject who was using corticosteroid injections during pollen season, but not outside of pollen season, stood out with the lowest amount of exhaled particle mass outside of pollen season and an increased amount during pollen season. The concentrations of SP-A and albumin in PEx did not change significantly between seasons or differ significantly between subjects with asthma and control subjects, as shown in Table 4. Although not significant ($P = 0\cdot10$, $n = 9$), there appeared to be a trend for the PEx albumin concentration to decrease during the pollen season in the subjects with asthma, with a median decrease of 0·5 wt%. Figure 3 shows that the amounts of SP-A and albumin in exhaled breath were strongly correlated to the PEx amount.

Exhaled nitric oxide

The median change in $FENO_{0\cdot27}$ during the pollen season was 1·3 ppb for the subjects with asthma and −0·1 ppb for control subjects. The seasonal changes in $FENO_{0\cdot05}$, $FENO_{0\cdot10}$ and $FENO_{0\cdot27}$ did not correlate significantly with changes in the exhaled mass of PEx per litre for either the subjects with asthma or control subjects. However, significant correlations were identified when the data for the subjects with asthma and control subjects were combined to increase statistical power; the most significant correlation with PEx was observed for $FENO_{0\cdot27}$ ($n = 23$ $r_s = -0\cdot57$, $P<0\cdot01$) and is presented in Fig. 4 ($FENO_{0\cdot05}$ $n = 25$ $r_s = -0\cdot46$ $P = 0\cdot02$, $FENO_{0\cdot10}$ $n = 24$ $r_s = -0\cdot35$ $P = 0\cdot09$).

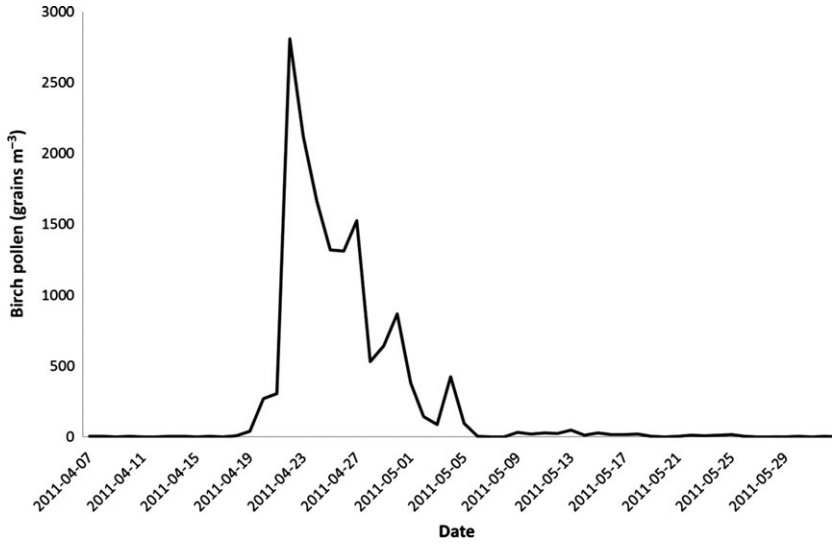

Figure 1 Birch pollen levels during the pollen season of 2011 in Gothenburg.

Table 2 Spirometry and $FENO_{0·05}$ data during and outside the pollen season.

	Subjects with asthma		Control subjects		Subjects with asthma versus control subjects	
	Out of season	Season	Out of season	Season	Out of season *P**	Season *P**
FVC (% of predicted normal value)†	105 (96–108)	105 (97–108)	116 (99–121)	106 (99–117)	0·21	0·46
FEV_1 (% of predicted normal value)†	92 (86–105)	94 (87–104)	103 (96–113)	98 (96–115)	0·12	0·16
FEV_1/FVC (%)†	78 (70–83)	78 (67–82)	79 (76–81)	79 (78–80)	0·84	0·54
ΔFEV_1 (% of predicted normal value)	3 (1–10)	5 (2–8)	1 (0–3)	1 (0–3)	**0·04**	**0·02**
$FENO_{0·05}$ (ppb)	20·3 (17–32)	54·1 (23–85)	13·3 (11–18)[a]	15·6 (10–20)[a]	**0·04**	**<0·01**

FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s.
Median values and interquartile range are presented.
*P-values refer to exact Wilcoxon–Mann–Whitney U test and significant P-values are in bold text.
†Prebronchodilator values; Δ FEV_1: $FEV_{1\%}$ of predicted normal value after bronchodilation minus $FEV_{1\%}$ of predicted normal value before bronchodilation; $FENO_{0·05}$, FENO measured at an exhalation flow of 0·05 l s^{-1}; ppb, parts per billion; $n = 13$.
[a]$n = 12$

Table 3 Data on exhaled particles during and outside of pollen season.

	Subjects with asthma Median (Q_1, Q_3)	Control subjects Median (Q_1, Q_3)
Out of season		
PEx (pg l^{-1})[a]	1250 (790, 1790)	1320 (1080, 2770)
PExNR (nr l^{-1})[a]	7450 (5080, 9860)	7520 (5970, 15 580)
SP-A (pg l^{-1})[a]	52 (35, 69)	28 (19, 172)
Albumin (pg l^{-1})[b]	90 (48, 112)	50 (43, 132)
SP-A/PEx (wt%)[a]	3·6 (3·0, 4·4)	2·6 (2·2, 3·4)
Albumin/PEx (wt%)[b]	4·1 (3·6, 5·9)	3·7 (3·3, 6·7)
Season		
PEx (pg l^{-1})[a]	900 (510, 1280)	1710 (840, 3540)
PExNR (nr l^{-1})[a]	5740 (2850, 8510)	9280 (4430, 17 850)
SP-A (pg l^{-1})[a]	37 (23, 42)	44 (22, 161)
Albumin (pg l^{-1})[b]	35 (28, 74)	87 (68, 167)
SP-A/PEx (wt%)[a]	3·2 (2·9, 3·5)	3·4 (1·9, 3·9)
Albumin/PEx (wt%)[b]	4·3 (3·3, 4·9)	5·5 (4·5, 6·0)

PEx, exhaled particles; pg l^{-1}, picograms per litre exhaled air; PExNR, particle number concentration based on particles with diameters of 0.41–4.55 μm; nr l^{-1}, number of particles per litre exhaled air; SP-A, surfactant protein A; wt%, weight per cent.
Median values are presented, with first and third quartiles in parentheses.
[a]$n = 13$.
[b]$n = 9$.

Discussion

This work has shown that the mass and number of particles in exhaled breath decreased during pollen season in subjects with asthma and birch pollen allergy but not in control subjects.

Exposure to natural birch pollen is well established as a way of inducing airway inflammation and can be used to study the inflammatory response of the epithelium in the airways (Tilles & Bardana, 1997; Kampe *et al.*, 2010). This was evident also in the present study according to the FENO results. Even though birch pollen grains are around 20 μm in diameter and are thus too large to penetrate efficiently into the small airways and directly cause inflammation, they can fragment and generate respirable allergens as small as 30 nm (Taylor *et al.*, 2007). In addition, exposure in the upper airways may elicit an immune response in the small airways via the trafficking of inflammatory mediators in the blood and lymphatic system (Braunstahl, 2005; D'Amato *et al.*, 2007). The pollen counts during spring 2011 would be expected to cause symptoms in the allergic subjects with asthma because even pollen counts below 100 grains m^{-3} have been found to increase allergic and respiratory symptoms (DellaValle *et al.*, 2012; Jantunen *et al.*, 2012).

The vast majority of exhaled particles are considered to be formed in the small airways by the mechanism of airway closure and subsequent reopening (Almstrand *et al.*, 2010). The observed decline in exhaled PEx mass during pollen season among the subjects with asthma may be due to either a reduced production of particles or an increased deposition of produced particles. Inflammation may induce prolonged closure of airways, reducing the number of airways participating in the repeated closure and reopening during inhalations. A study by Jarjour & Enhorning (1999) used bronchoalveolar lavage to compare surfactant properties in allergen-challenged and saline-challenged lung segments. They found that in subjects with asthma, the lavage fluid recovered from allergen-challenged segments indicated a reduced capability to maintain airway patency compared to the control segments. It was concluded that allergen-induced inflammation can lead to some blocking of the terminal airways by liquid columns. It is also conceivable that inflammation could change the geometry of the airways or the breathing patterns of the subjects with asthma during the pollen season, leading to an increased deposition of produced particles. Altered concentrations of SP-A or albumin in PEx could be associated with changes in RTLF surface tension and/or film thickness, both of which are expected to influence surfactant properties and particle

Table 4 Interseasonal changes in exhaled particles.

	Subjects with asthma		Control subjects	
	Median (Q_1, Q_3)	**$P^{\dagger}$ values**	**Median (Q_1, Q_3)**	**$P^{\dagger}$ values**
Seasonal change				
△PEx (pg l^{-1})[a]	−290 (−420, −160)	**<0·01***	520 (−230, 780)	0·74*
△PExNR (nr l^{-1})[a]	−1790 (−2820, −650)	**0·04***	840 (−1860, 4360)	0·95*
△SP-A (pg l^{-1})[a]	−12 (−39, 4)	0·17*	0 (−9, 10)	0·95*
△Albumin (pg l^{-1})[b]	−32 (−43, −13)	**<0·01***	27 (−4, 35)	0·10*
△SP-A/PEx (wt%)[a]	−0·1 (−0·7, 0·3)	0·79	−0·2 (−0·5, 0·4)	0·74
△Albumin/PEx (wt%)[b]	−0·5 (−1·1, 0·1)	0·10	0·5 (−0·7, 1·5)	0·50

Median values are presented, with first and third quartiles in parentheses. Significant P-values are in bold text.
△, (in-season value − out-of-season value); PEx, exhaled particles; pg l^{-1}, picograms per litre exhaled air; PExNR, particle number concentration based on particles with diameters of 0.41–4.55 μm; nr l^{-1}, number of particles per litre exhaled air; SP-A, surfactant protein A; wt%, weight per cent.
[a]n = 13.
[b]n = 9.
*P-value is calculated from log-transformed data.
[†]Two-sided Wilcoxon signed rank-sum test for seasonal change of paired data.

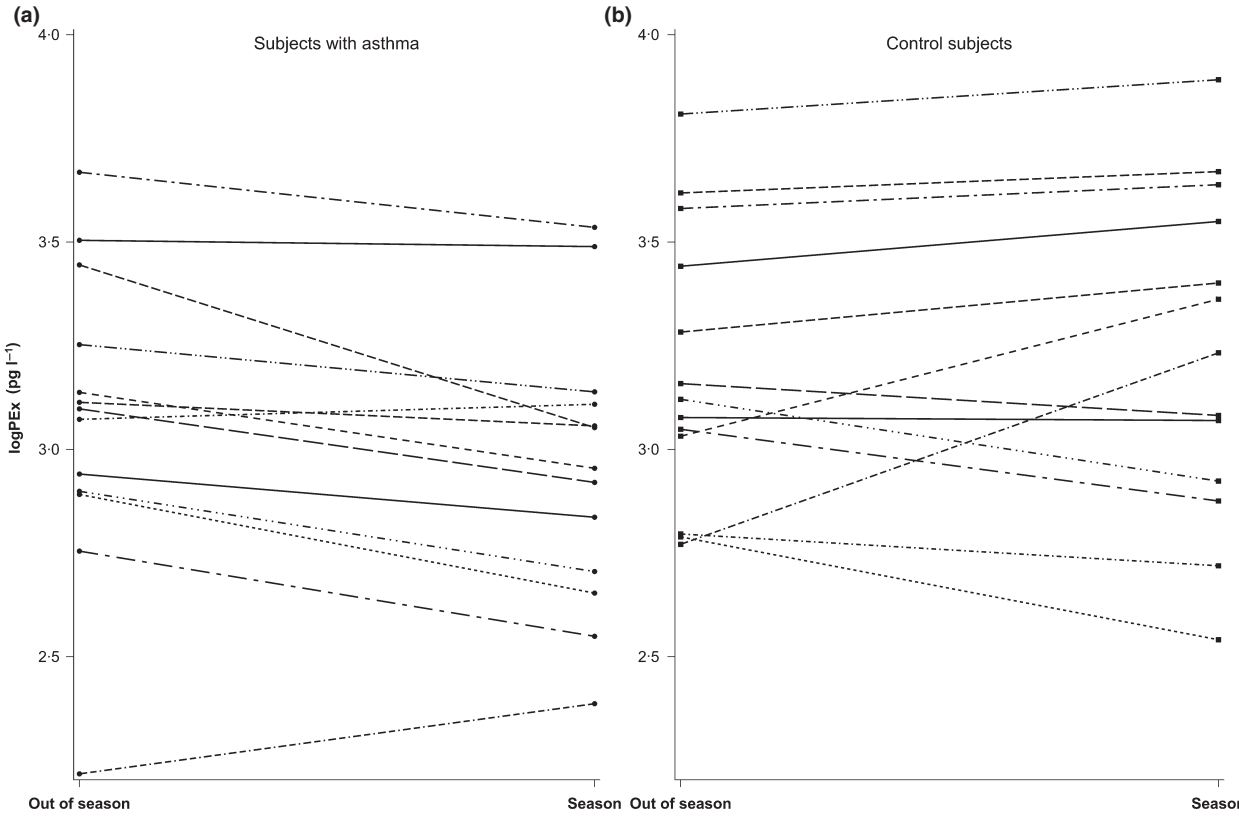

Figure 2 Interseasonal differences in mass of particles (PEx) per litre exhaled air for (a) subjects with asthma and (b) control subjects. Each line in the individual plots represents results for a single subject. log, decadic logarithm; PEx, exhaled particles; pg l^{-1}, picograms per litre exhaled air.

formation (McEntee & Mysels, 1969; Widdicombe, 2002; Holmgren & Ljungstrom, 2012). However, no significant interseasonal differences in the SP-A and albumin contents of the PEx were observed in this work. It is possible that the inflammation induced by birch pollen exposure was mild and did not change the concentrations of SP-A or albumin in the lower airways sufficiently to be measured with the used methods. It is also possible that the pollen-induced inflammation is localized in more central airways with higher pollen exposure that are not sampled by the PEx method. Although not observed in this work, other studies have demonstrated that allergen exposure can change SP-A concentrations in subjects

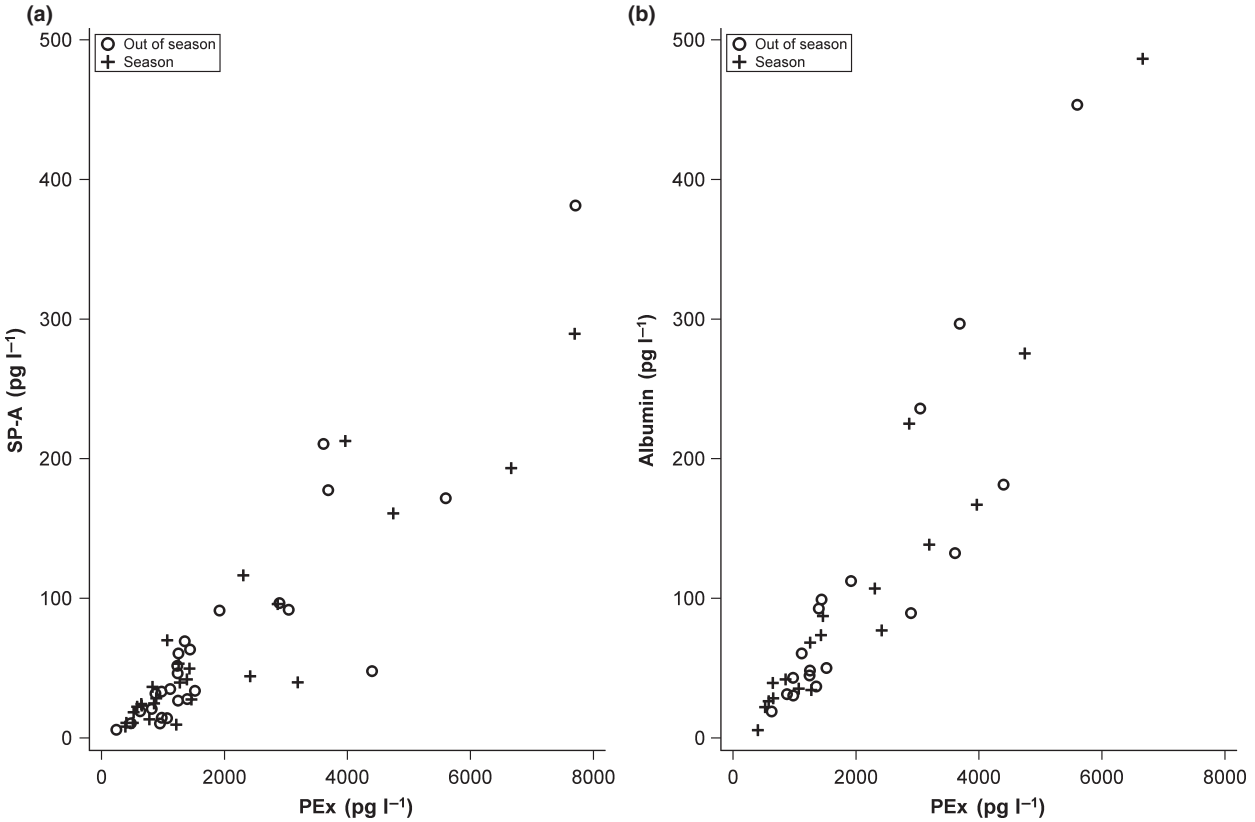

Figure 3 Relationships between the mass of particles per litre exhaled air and the concentrations of (a) surfactant protein A and (b) albumin in the exhaled air. The plots demonstrate that PEx is linearly related to both SP-A (R^2 = 0·81, n = 52) and albumin (R^2 = 0·85, n = 36) concentrations. The results for the subjects with asthma and control subjects were pooled, and the results for in- and out-of-season measurements were combined when computing the coefficient of determination. SP-A, surfactant protein A; PEx, exhaled particles; pg l^{-1}, picograms per litre exhaled air.

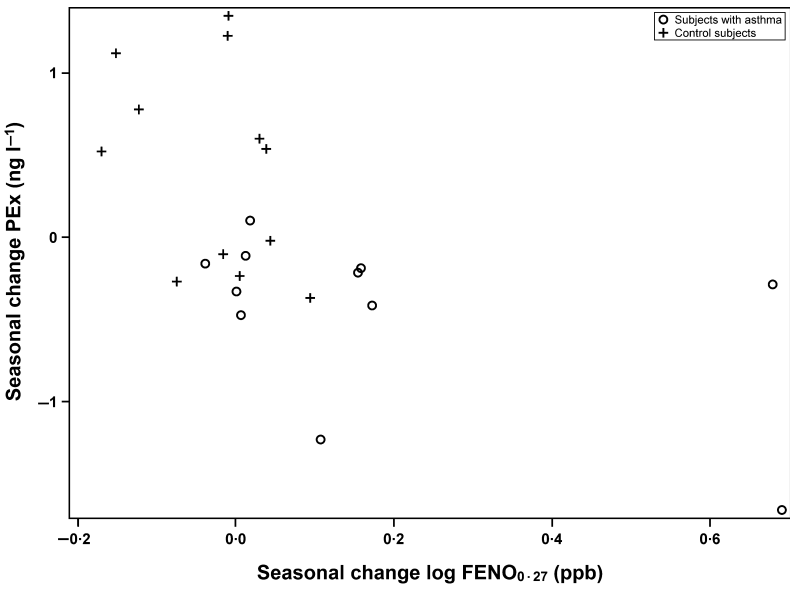

Figure 4 Relationship between the seasonal changes in the exhaled particle mass and FENO. Seasonal changes are defined as the difference between the in- and out-of-season values for both variables. log, decadic logarithm; $FENO_{0·27}$, FENO measured at an exhalation flow of 0·27 l s^{-1} (n = 23); ppb, parts per billion; PEx, exhaled particles; ng l^{-1}, nanograms per litre exhaled air.

with asthma (Hohlfeld *et al.*, 2002; Kishore *et al.*, 2005; Erpenbeck *et al.*, 2006). Erpenbeck *et al.* (2006) found that allergen challenge reduced the SP-A levels in bronchoalveolar lavage fluid from subjects with asthma but not that from healthy controls (Jarjour & Enhorning, 1999). A study by Koopmans *et al.* (2004) found that allergen challenge of the

airways increased serum levels of SP-A. These observations are consistent with an increase in permeability between the airway–vascular barriers and an SP-A leakage that is not compensated for by an increase in production. A compromised airway–vascular barrier may also lead to an influx of serum proteins and inflammatory mediators into the airways as was demonstrated by Khor *et al.* (2009), who found that increasing inflammation and deteriorating asthma control were associated with an increase of albumin levels in bronchoalveolar lavage fluid. It is difficult to compare results from this work to previous studies as there are uncertainties regarding the different subjects' exposure levels as well as the degree and type of inflammation that they experienced. This may also explain some of the conflicting results obtained in previously published studies on bronchoalveolar lavage (Cheng *et al.*, 2000; Erpenbeck *et al.*, 2006).

As a marker of inflammation in small airways, FENO was measured for both subjects with asthma and control subjects. In principle, a correlation between an increase in FENO and a decline in exhaled PEx (ng l^{-1}) would support an association between the exhaled PEx amount and inflammation in the lower airways. No statistically significant correlation between FENO (ppb) and PEx (ng l^{-1}) was found for subjects with asthma. For exploratory purposes, the FENO data for the subjects with asthma and control subjects were combined to increase statistical power. Analysis of the combined data revealed a significant correlation between FENO and PEx, especially for $FENO_{0.27}$. However, this finding should be interpreted with caution as the number of observations was low and the change between seasons was small.

The main limitations of this work are the limited number of participating subjects with asthma and the uncertainties regarding the subjects' individual pollen exposure levels and degrees of small airway inflammation. Ideally, the study would have incorporated tests on the physiology of the subjects' small airways to verify the presence of inflammation and/or increases in ventilation heterogeneity. In future studies, the variability of the results obtained using the PExA® method could be reduced by sampling a constant predetermined PEx mass from all subjects rather than a fixed volume of exhaled breath. By selecting an appropriate PEx amount for the subsequent chemical analysis, it would be possible to reduce the number of samples whose concentrations of target analytes are close to the limits of the chosen analytical technique; the use of such dilute samples tends to increase the variability in the results of chemical analysis.

Conclusion

Birch pollen exposure may reduce the extent of small airway closure and reopening during the pollen season but has negligible effect on the concentrations of SP-A and albumin in the RTLF of the small airways of subjects with mild intermittent asthma.

Funding statement

This research was funded by the Swedish Research Council Formas (Stockholm, Sweden; project No. 2009-789), the Swedish Heart-Lung Foundation (Stockholm, Sweden; project No. 20110353) and the Västra Götaland county council ALF-fund (distributed to Anna-Carin Olin).

Acknowledgments

The authors would like to thank Marianne Andersson and Annika Claesson for coordinating the study and collecting the samples (Occupational and Environmental Medicine, Sahlgrenska Academy at Gothenburg University, Gothenburg, Sweden). We also wish to acknowledge Sees-editing Ltd for editing of the manuscript.

Conflict of interest

The authors Per Larsson, Mona Lärstad, Björn Bake, Anna Bredberg, Ann-Charlotte Almstrand, Ekaterina Mirgorodskaya and Anna-Carin Olin are shareholders in PExA®AB (http://pexa.se).

References

Almstrand AC, Ljungstrom E, Lausmaa J, *et al.* Airway monitoring by collection and mass spectrometric analysis of exhaled particles. *Anal Chem* (2009); **81**: 662–668.

Almstrand AC, Bake B, Ljungstrom E, *et al.* Effect of airway opening on production of exhaled particles. *J Appl Physiol (1985)* (2010); **108**: 584–588.

Bake B, Viklund E, Olin AC. Effects of pollen season on central and peripheral nitric oxide production in subjects with pollen asthma. *Respir Med* (2014); **108**: 1277–1283.

Bernhard W, Haagsman HP, Tschernig T, *et al.* Conductive airway surfactant: surface-tension function, biochemical composition, and possible alveolar origin. *Am J Respir Cell Mol Biol* (1997); **17**: 41–50.

Braunstahl GJ. The unified immune system: respiratory tract-nasobronchial interaction mechanisms in allergic airway disease. *J Allergy Clin Immunol* (2005); **115**: 142–148.

Bredberg A, Gobom J, Almstrand AC, *et al.* Exhaled endogenous particles contain lung proteins. *Clin Chem* (2012); **58**: 431–440.

Cheng G, Ueda T, Numao T, *et al.* Increased levels of surfactant protein A and D in bronchoalveolar lavage fluids in patients with bronchial asthma. *Eur Respir J* (2000); **16**: 831–835.

D'Amato G, Cecchi L, Bonini S, *et al.* Allergenic pollen and pollen allergy in Europe. *Allergy* (2007); **62**: 976–990.

DellaValle CT, Triche EW, Leaderer BP, *et al.* Effects of ambient pollen concentrations on frequency and severity of asthma symptoms among asthmatic children. *Epidemiology* (2012); **23**: 55–63.

Erpenbeck VJ, Schmidt R, Gunther A, *et al.* Surfactant protein levels in bronchoalveolar lavage after segmental allergen challenge in patients with asthma. *Allergy* (2006); **61**: 598–604.

Haslbeck K, Schwarz K, Hohlfeld JM, *et al.* Submicron droplet formation in the human lung. *J Aerosol Sci* (2010); **41**: 429–438.

Hermans C, Bernard A. Lung epithelium-specific proteins: characteristics and potential applications as markers. *Am J Respir Crit Care Med* (1999); **159**: 646–678.

Hohlfeld JM, Erpenbeck VJ, Krug N. Surfactant proteins SP-A and SP-D as modulators of the allergic inflammation in asthma. *Pathobiology* (2002); **70**: 287–292.

Holmgren H, Ljungstrom E. Influence of film dimensions on film droplet formation. *J Aerosol Med Pulm Drug Deliv* (2012); **25**: 47–53.

Holmgren H, Ljungstrom E, Almstrand AC, *et al.* Size distribution of exhaled particles in the range from 0.01 to 2.0 mu m. *J Aerosol Sci* (2010); **41**: 439–446.

Jantunen J, Saarinen K, Rantio-Lehtimaki A. Allergy symptoms in relation to alder and birch pollen concentrations in Finland. *Aerobiologia* (2012); **28**: 169–176.

Jarjour NN, Enhorning G. Antigen-induced airway inflammation in atopic subjects generates dysfunction of pulmonary surfactant. *Am J Respir Crit Care Med* (1999); **160**: 336–341.

Johnson GR, Morawska L. The mechanism of breath aerosol formation. *J Aerosol Med Pulm Drug Deliv* (2009); **22**: 229–237.

Kampe M, Janson C, Stalenheim G, *et al.* Experimental and seasonal exposure to birch pollen in allergic rhinitis and allergic asthma with regard to the inflammatory response. *Clin Respir J* (2010); **4**: 37–44.

Khor YH, Teoh AKY, Lam SM, *et al.* Increased vascular permeability precedes cellular inflammation as asthma control deteriorates. *Clin Exp Allergy* (2009); **39**: 1659–1667.

Kishore U, Bernal AL, Kamran MF, *et al.* Surfactant proteins SP-A and SP-D in human health and disease. *Archivum immunologiae et therapiae experimentalis* (2005); **53**: 399–417.

Kishore U, Greenhough TJ, Waters P, *et al.* Surfactant proteins SP-A and SP-D: structure, function and receptors. *Mol Immunol* (2006); **43**: 1293–1315.

Knowles MR, Robinson JM, Wood RE, *et al.* Ion composition of airway surface liquid of patients with cystic fibrosis as compared with normal and disease-control subjects. *J Clin Investig* (1997); **100**: 2588–2595.

Koopmans JG, van der Zee JS, Krop EJ, *et al.* Serum surfactant protein D is elevated in allergic patients. *Clin Exp Allergy* (2004); **34**: 1827–1833.

Larsson P, Mirgorodskaya E, Samuelsson L, *et al.* Surfactant protein A and albumin in particles in exhaled air. *Respir Med* (2012); **106**: 197–204.

McEntee WR, Mysels KJ. Bursting of soap films. I. An experimental study. *J Phys Chem* (1969); **73**: 3018–3028.

Miller MR, Hankinson J, Brusasco V, *et al.* Standardisation of spirometry. *Eur Respir J* (2005); **26**: 319–338.

Nicholson JP, Wolmarans MR, Park GR. The role of albumin in critical illness. *Br J Anaesth* (2000); **85**: 599–610.

Perez-Gil J. Structure of pulmonary surfactant membranes and films: the role of proteins and lipid-protein interactions. *Biochim Biophys Acta* (2008); **1778**: 1676–1695.

Quanjer PH, Tammeling GJ, Cotes JE, *et al.* Lung volumes and forced ventilatory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J Suppl* (1993); **16**: 5–40.

Schurch S, Lee M, Gehr P. Pulmonary surfactant – surface-properties and function of alveolar and airway surfactant. *Pure Appl Chem* (1992); **64**: 1745–1750.

Schwarz K, Biller H, Windt H, *et al.* Characterization of exhaled particles from the healthy human lung–a systematic analysis in relation to pulmonary function variables. *J Aerosol Med Pulm Drug Deliv* (2010); **23**: 371–379.

Schwarz K, Biller H, Windt H, *et al.* Characterization of exhaled particles from the human lungs in airway obstruction. *J Aerosol Med Pulm Drug Deliv* (2015); **28**: 52–58.

Taylor PE, Jacobson KW, House JM, *et al.* Links between pollen, atopy and the asthma epidemic. *Int Arch Allergy Immunol* (2007); **144**: 162–170.

Tilles SA, Bardana EJ Jr. Seasonal variation in bronchial hyperreactivity (BHR) in allergic patients. *Clin Rev Allergy Immunol* (1997); **15**: 169–185.

Widdicombe JH. Regulation of the depth and composition of airway surface liquid. *J Anat* (2002); **201**: 313–318.

Wright JR. Immunoregulatory functions of surfactant proteins. *Nat Rev Immunol* (2005); **5**: 58–68.