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Low Levels of Exhaled Surfactant Protein A Associated With BOS After Lung Transplantation

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Background. There is no clinically available marker for early detection or monitoring of chronic rejection in the form of bronchiolitis obliterans syndrome (BOS), the main long-term complication after lung transplantation. Sampling and analysis of particles in exhaled air is a valid, noninvasive method for monitoring surfactant protein A (SP-A) and albumin in the distal airways. **Methods.** We asked whether differences in composition of exhaled particles can be detected when comparing stable lung transplant recipients (LTRs) (n = 26) with LTRs who develop BOS (n = 7). A comparison between LTRs and a matching group of healthy controls (n = 33) was also conducted. Using a system developed in-house, particles were collected from exhaled air by the principle of inertial impaction before chemical analysis by immunoassays. **Results.** Surfactant protein A in exhaled particles and the SP-A/albumin ratio were lower ($P = 0.002$ and $P = 0.0001$ respectively) in the BOS group compared to the BOS-free group. LTRs exhaled higher amount of particles ($P < 0.0001$) and had lower albumin content ($P < 0.0001$) than healthy controls. **Conclusions.** We conclude that low levels of SP-A in exhaled particles are associated with increased risk of BOS in LTRs. The possibility that this noninvasive method can be used to predict BOS onset deserves further study with prospective and longitudinal approaches.

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Lung transplantation is the only treatment option for many end-stage lung diseases. Results have improved over the years; however, the long-term prognosis is still poor with only approximately 55% of the patients surviving more than 5 years.¹

Chronic rejection is the main survival limiting complication. Examination of rejected lung tissue show histopathologic changes of obliterative bronchiolitis (OB), characterized by a chronic fibroproliferative process in the small airways leading to airflow limitation and subsequently a progressive loss of lung function.² Histological confirmation of OB with bronchoscopy and transbronchial biopsies is difficult to obtain due to the patchy appearance of OB and limited biopsy sizes. Hence, the term bronchiolitis obliterans syndrome (BOS) that

refers to the clinical diagnosis of chronic rejection based on spirometry findings was established.³ Bronchiolitis obliterans syndrome is characterized by an obstructive ventilatory defect. Over the last years, it has been recognized that additional entities, such as restrictive allograft syndrome, can lead to chronic decline in forced expiratory volume in 1 second (FEV₁) after lung transplantation. The term chronic lung allograft dysfunction has been introduced to cover all forms of graft dysfunction.⁴ Bronchiolitis obliterans syndrome is associated with impaired innate defenses within the lung.^{5,6} Surfactant protein A (SP-A), locally produced by alveolar type II cells is a component of the lung allograft's innate defenses.⁷⁻¹⁰ A significant reduction of bronchoalveolar lavage (BAL) SP-A content has been found in BOS patients compared with stable lung transplant recipients (LTRs).¹¹

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These senior authors contributed equally to this study (G.C.R., A.-C.O.).

All authors participated in data interpretation, reviewed, and approved the final article. P.E. wrote the main text of the article and prepared all figures. G.R.,

A.-C.O., A.-C.A., E.M., and P.E. conceived and designed the experiments. A.-C.A. and E.V. performed the clinical examinations including sampling of PEx. O.H., P.E., G.R., E.M., and E.V. analyzed the data. P.L. and E.M. set up the immunodetection assays and analyzed all samples. A.-C.O., A.-C.A., and P.L. developed the sampling technique. O.H. was responsible for the statistical evaluation.

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The particles in exhaled air (PExA) method is a novel, non-invasive method for monitoring the airways developed at the Department of Occupational and Environmental Medicine, University of Gothenburg, Sweden. The sample, endogenous particles (PEx), are collected using an inertial impactor with subsequent chemical analysis.¹² The particles originate from the epithelial lining fluid in the small airways and their protein and lipid profile is similar to that of BAL.¹³ It has previously been demonstrated that collection of particles is a valid method for quantifying exhaled SP-A originating from epithelial lining fluid in humans.¹⁴⁻¹⁶ There are presently no clinically useful markers for early detection and/or monitoring of BOS. This is unfortunate since early diagnosis can lead to augmented immunosuppressive treatment and increased survival rates.¹⁷ The development of alternative noninvasive diagnostic methods that enable repeated measurements would greatly facilitate our understanding of the biological processes occurring in the distal airways during allograft rejection.

The aim of this exploratory study was to investigate if SP-A in PEx could be used as an early biomarker for BOS. We also evaluated whether differences in particle composition can be observed when comparing healthy subjects to LTRs.

MATERIALS AND METHODS

Study Subjects and Design

Lung transplant recipients were included consecutively from November 2012 to May 2014 at the time for their scheduled clinical assessment at 6 months or more after transplantation. Inclusion criteria were stable condition after lung transplantation, freedom from BOS at the prior visit and absence of significant infection at the study visit.

Surgical procedures, immunosuppression therapy, clinical follow-up and routine transbronchial biopsy, and BAL specimen analysis for infectious agents were performed according

to the lung transplant protocol of Sahlgrenska University Hospital.¹⁸ The morphologic evaluation of OB followed the standard recommendations and BOS was defined as an irreversible decline in FEV₁ of at least 20% of the baseline value (defined as the average maximum FEV₁ value of 2 consecutive measurements >30 days apart during the first postoperative year).³

Lung function assessment and particle collection were performed before bronchoscopy. Each individual was followed up for 12 months after the study visit and then categorized as BOS or non-BOS.

Healthy subjects participating in the Gothenburg part of the European Community Respiratory Health Survey, matched for age and sex were used as controls. Subjects were selected if they had neither chronic obstructive pulmonary disease (defined as a postbronchodilator FEV₁/forced vital capacity (FVC) ratio <0.7) nor asthma (excluding those with physician-diagnosed asthma and those reporting asthma symptoms or taking asthma medication) and were nonsmokers. The matching procedure chose healthy controls of the same sex and with the least possible age difference.

The study design was approved by the Regional Ethics Review Committee of the University of Gothenburg (diary no. 390-06, 472-07). All subjects gave their informed written consent.

Sampling of PEx

Exhaled endogenous particles were collected with the PExA method, as previously described, with small modifications (Figure 1).¹¹ The exhaled particles were sampled on a Teflon membrane (FHLC02500, Merck Millipore, Billerica, MA) and the total number of exhaled particles and total volume of the exhaled breath were recorded. The total mass of the collected samples was calculated based on the number and size of the collected particles, assuming them to be spherical and have a density of 1000 kg/m³.

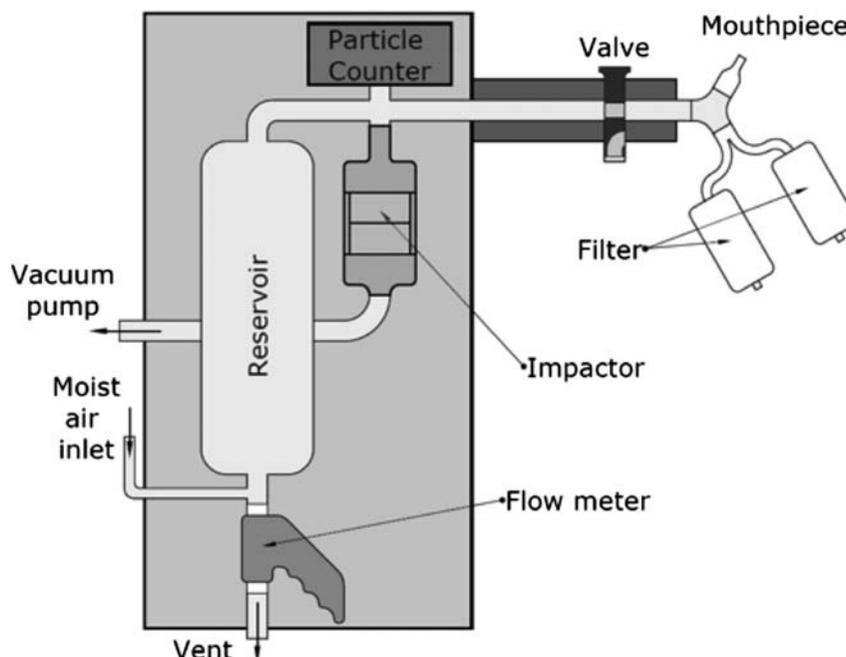


FIGURE 1. The subject exhales via a mouthpiece and a directional valve into a thermostated box (36°C) containing an exhaled air reservoir, a Grimm 1.108 optical particle counter (Grimm Aerosol Technik GmbH & Co, Ainring, Germany), and an impactor (3-stage PM 10 Impactor, Dekati Ltd., Tampere, Finland). Using a vacuum pump the exhaled air containing particles is drawn through the impactor and the particles are collected by impaction according to their size on the hydrophilic Teflon membrane. Figure courtesy of Anna Bredberg.

All subjects wore a nose clip throughout the sampling procedure. Before PEx sampling, subjects inhaled HEPA-filtered (Whatman Inc., NJ) air for 2 minutes to remove particles originating from ambient air. During sampling, the subjects performed a standardized breathing maneuver allowing for airway closure and reopening¹⁹: (1) exhalation to residual volume, (2) rapid inhalation to total lung capacity, (3) deep relaxed exhalation. Only the last exhalation was sampled in the instrument. Between breathing maneuvers, the study subject breathed particle-free air tidally. The procedure was repeated until a target volume of 60 L of exhaled air or a maximum sampling time of 30 minutes was reached. After collection, the Teflon membrane was transferred to a polypropylene vial and stored at -80°C until analysis.

SP-A and Albumin Enzyme-Linked Immunosorbent Assay (ELISA)

Solvent Preparation

The following solvents were prepared and used for sample preparation and analysis. *Extraction buffer* prepared as phosphate-buffered saline (PBS) 10 mM Na Phosphate, 0.15 M NaCl, containing 1% bovine serum albumin, w/v, and 0.05% Tween-20, v/v. (ELISA) *sample diluents* prepared according to ELISA manufacturer's recommendation.

Assay buffers were prepared by mixing *extraction buffer* and the corresponding *ELISA sample diluent* in the ratio 1:2, v:v.

Particle Extraction

Particles were extracted from Teflon membranes using extraction buffer. To each sample, 140 μL of the extraction buffer was added, followed up by 60-minute shaking at 400 rpm and 37°C in a thermomixer (Thermomixer comfort, Eppendorf; Eppendorf AG, Hamburg, Germany). Three polypropylene vials, each containing 40 μL of extract, were prepared and stored at -20°C before analysis. One vial was used for SP-A assay, another for the albumin assay, and the third vial was maintained as reserve sample.

Sample Preparation

Before immunoassays, samples were thawed to room temperature and diluted 3 times with provided ELISA sample diluents. Samples with mass over 450 ng but below 1000 ng were further diluted 3 times with assay buffer. All samples with mass over 1000 ng were diluted 9 times with assay buffer.

SP-A ELISA

Surfactant protein A in extracted particle samples was quantified using a human SP-A ELISA kit (RD191139200R, BioVendor, Czech Republic). The assays were performed according to the manufacturer's instructions, with minor modifications to the buffer composition and incubation time. All calibrants and controls were prepared and assayed in the same assay buffer as particle samples. The plate incubation time was extended from 2 to 3 hours. The absorbance was read at 450 nm by a plate-reader from BioTek ELx-808UI (Highland Park, MI). The limit of quantification was 0.5 ng mL^{-1} as determined by precision profile at 15% coefficient of variation (CV). The CV for the intra assay variability determined from duplicate sample analysis was 4.6%.

Albumin ELISA

Albumin was quantified using human albumin ELISA kit (E-80AL, Immunology Consultants Laboratory, Inc., USA) according to the manufacturer's instructions, with minor modifications to the buffer composition and incubation time. All calibrants and controls were prepared and assayed in the same assay buffer as particle samples. The plate incubation time was extended from 1 to 1.5 hours. The absorbance was read at 450 nm. Limit of quantification was 0.9 ng mL^{-1} as determined by precision profile at 15% CV. The CV for the intra-assay variability determined from duplicate sample analysis was 2.7%.

Calculation of Protein Concentrations

Concentration of proteins in exhaled particles (mg mL^{-1} of particles) was calculated by dividing determined protein mass with the sampled volume of particles. Particle volume was calculated based on the recorded particle size. The protein concentration in mg mL^{-1} is corresponding to weight percent protein ($\text{wt}\%$) $\times 10$.

Statistical Analysis

Mean and median values are reported as appropriate. Mann-Whitney *U* tests were used to test differences between groups. Statistical analyses were carried out with SAS, version 9.4 (SAS Statistical package, Cary, NC). Each patient was matched to 1 healthy control using the *vmatch* SAS macro.²⁰

The analyzed data set has missing values due to technical problems while recording particle data. Particle mass concentrations have missing data for 2 patients in the BOS group and 3 patients in the non-BOS group. This occurred due to a faulty particle counter output, faulty exhaled volume output, or both. Protein concentrations in exhaled particles have 1 missing value for a patient in the non-BOS group due to faulty particle counter output.

RESULTS

Study Subjects and Design

Thirty-six patients accepted to participate. Three patients had significant infection (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Aspergillus* species) and were therefore not included in the final analysis. Two patients had a serological reactivation of cytomegalovirus (polymerase chain reaction-cytomegalovirus) without clinical symptoms. Five patients were transplanted with a single lung (pretransplant diagnosis; chronic obstructive pulmonary disease, $n = 3$; idiopathic pulmonary fibrosis, $n = 1$; lymphangioliomyomatosis, $n = 1$). Seven patients had received lungs treated with ex vivo lung perfusion (EVLP), a fairly novel method reconditioning lungs that initially are not accepted as donor organs.²¹ Of the 33 patients included in the study, 32 patients were followed up for 12 months after PEx collection. One patient died 7 months after the study visit, not related to BOS development. Two patients were diagnosed with BOS at the study visit and another 5 developed BOS during the follow up period (3, 6, 6, 6, and 12 months after study inclusion respectively). No patient was diagnosed with restrictive allograft syndrome. The study visit was performed between 6 and 36 months (median, 9 months) after the transplantation. The median age difference of matched pairs (LTR—healthy control) was 0.19 years and the maximum age difference was 5 years. The study subject characteristics are presented in Table 1.

TABLE 1.**Study subject characteristics**

	non-BOS (n = 26)	BOS (n = 7)	Controls (n = 33)
Male/female (n)	15/11	2/5	17/16
Mean age, y	55,4	57,6	56
Type of operation (SL/BL)	3/23	2/5	
EVLP (n)	6	1	
Preoperative diagnosis (n)			
COPD	10	4	
IPF	5	1	
A1AT	4	2	
CF	4		
Other	3		

SL, single lung; BL, bilateral lung; COPD, chronic obstructive pulmonary disease; IPF, idiopathic pulmonary fibrosis; A1AT, α -1-antitrypsin deficiency; CF, cystic fibrosis.

Amount of Exhaled Particles

The amount of PEx collected per liter exhaled air (ie, mass concentration) was markedly higher in the LTRs (BOS + non-BOS) (median, 8.0; range, 2.8-65.3 $\text{ng} \times \text{L}^{-1}$) compared with the healthy controls (median, 1.8; range, 0.4-19.4 $\text{ng} \times \text{L}^{-1}$) ($P < 0.0001$). There was also a significant difference between the BOS (median, 19.3; range, 3.2-65.3 $\text{ng} \times \text{L}^{-1}$), and the

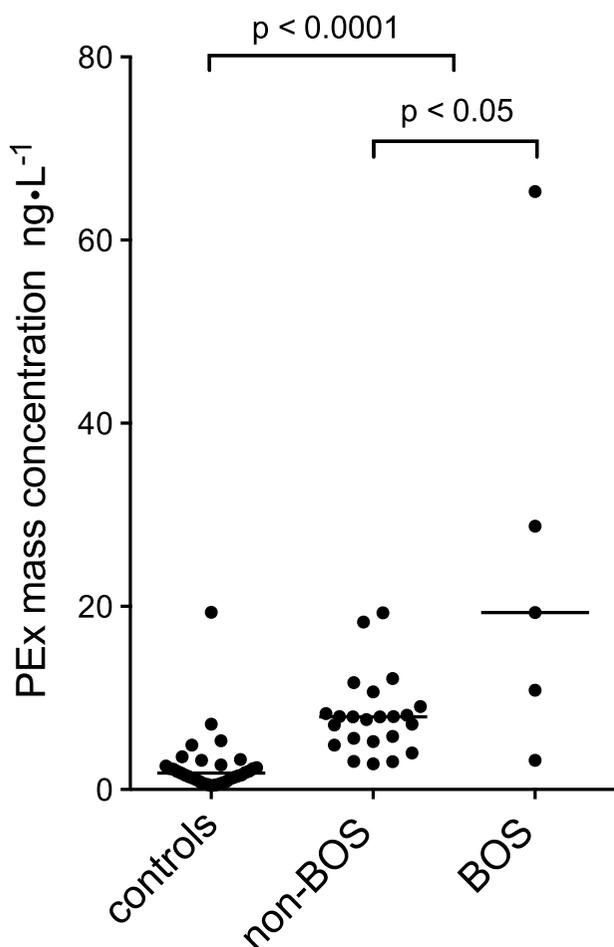


FIGURE 2. Amount of PEx (ng) collected per liter exhaled air from healthy controls (n = 33), stable lung transplant recipients (non-BOS) (n = 23) and patients that developed BOS (n = 5). Bars represent the median.

non-BOS group (median, 7.9; range, 2.8-19.3 $\text{ng} \times \text{L}^{-1}$) ($P < 0.05$) (Figure 2).

Surfactant protein A

Surfactant protein A in PEx was significantly lower in the BOS group (median, 18; range, 7-32 $\text{mg} \times \text{mL}^{-1}$) compared with the non-BOS group (median, 30; range, 20-57 $\text{mg} \times \text{mL}^{-1}$) ($P = 0.002$) but there was no difference between LTRs (BOS + non-BOS) (median, 28; range, 7-57 $\text{mg} \times \text{mL}^{-1}$) and healthy controls (median, 29; range, 15-57 $\text{mg} \times \text{mL}^{-1}$) ($P = \text{ns}$) (Figure 3).

Albumin

Albumin in PEx was significantly higher in the healthy controls (median, 64; range, 46-123 $\text{mg} \times \text{mL}^{-1}$) than in the LTRs (BOS + non-BOS) (median, 47; range, 20-144 $\text{mg} \times \text{mL}^{-1}$) ($P < 0.0001$), but there was no difference between the BOS (median, 46; range, 20-144 $\text{mg} \times \text{mL}^{-1}$) and the non-BOS group (median, 47; range, 22-97 $\text{mg} \times \text{mL}^{-1}$) groups ($P = \text{ns}$) (Figure 4).

SP-A/Albumin Ratio

The SP-A/Albumin ratio was lower in the BOS group (median, 0.35; range, 0.18-0.45) than in the non BOS group (median, 0.74; range, 0.26-1.6) ($P = 0.0001$) but there was no significant difference between LTRs (BOS + non-BOS) (median, 0.61; range, 0.18-1.6) and healthy controls (median, 0.45, range, 0.18-1.1) ($P = \text{ns}$) (Figure 5).

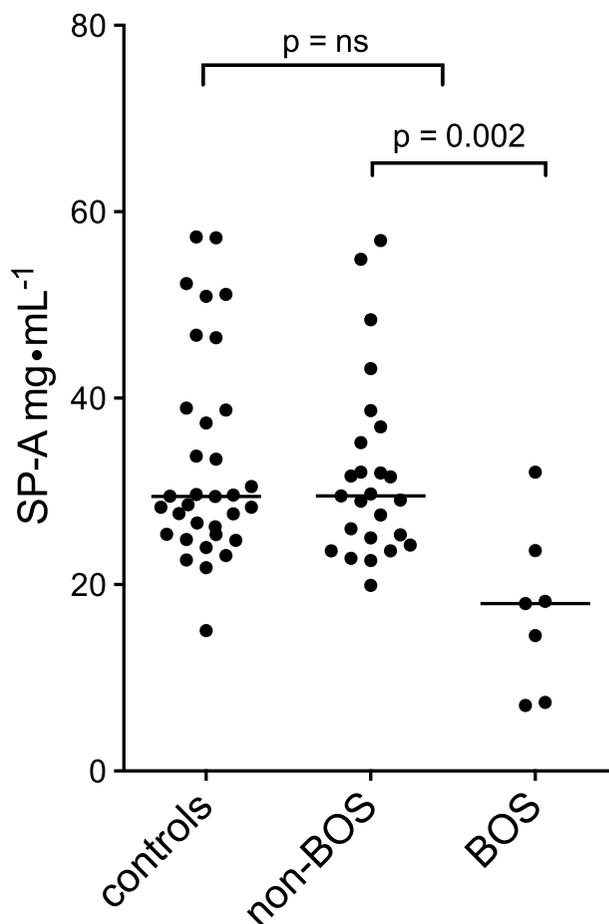


FIGURE 3. SP-A ($\text{mg} \times \text{mL}^{-1}$) measured by ELISA in PEx from healthy controls (n = 33), stable lung transplant recipients (non-BOS) (n = 25) and patients that developed BOS (n = 7). Bars represent the median.

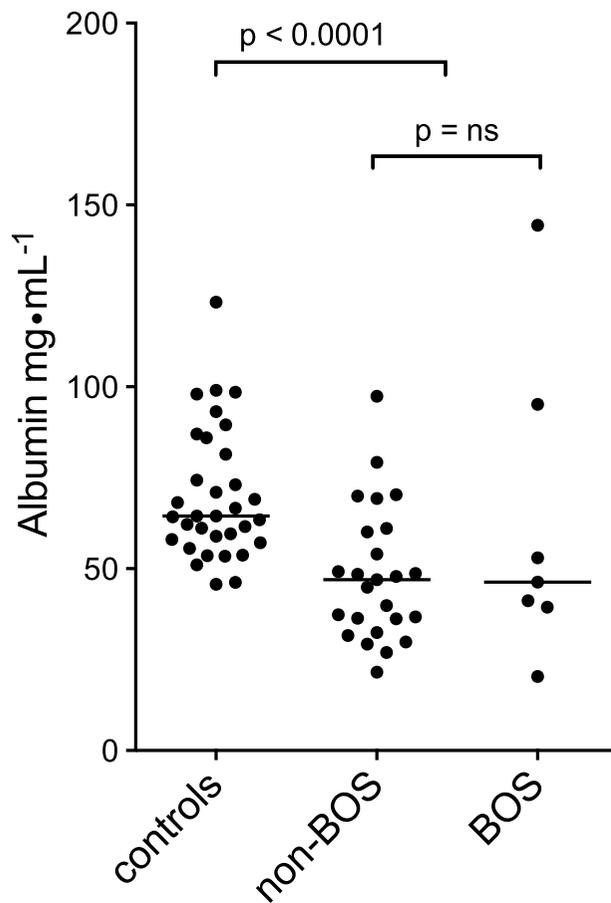


FIGURE 4. Albumin (mg mL^{-1}) measured by ELISA in PEx from healthy controls ($n = 33$), stable lung transplant recipients (non-BOS) ($n = 25$) and patients that developed BOS ($n = 7$). Bars represent the median.

PEx Outcome in Relation to Age, Gender and EVLP in LTRs

We found no significant associations for PEx outcomes (PEx mass concentration, SP-A, concentration in PEx, albumin concentration in PEx and SP-A/albumin ratio) with age, sex, or EVLP (data not shown). To avoid the influence of BOS only the BOS-free group ($n = 26$) was included in the analysis.

DISCUSSION

In this exploratory study, SP-A concentration in PEx was significantly lower in patients developing BOS (2 patients diagnosed at the study visit, 5 during the follow-up period) compared with stable LTRs. The SP-A/albumin ratio was also lower in the BOS group. This indicates that SP-A in PEx could be a more sensitive diagnostic tool for BOS detection than spirometry. The results are in line with earlier findings in BAL samples by Meloni et al¹¹ who found a lower SP-A content in BOS patients compared with stable LTRs. The amount of SP-A in BAL obtained from LTRs who developed BOS was also low in the pre-BOS period, which may suggest that SP-A is involved in BOS pathogenesis.

A major mechanism in PEx formation is airway reopening after closure in the distal bronchioles.¹⁹ To ensure that the particles exhaled by each individual patient were formed by the same mechanism, we used a specific breathing maneuver

developed to maximize airway closure and reopening and thereby the number of exhaled particles from the distal airways. Why LTRs, and in particular LTRs developing BOS, exhaled higher number of particles than age matched controls is not known but might be explained by a higher degree of airway closure and reopening. This is in line with our previous results of small airway collapse in LTRs using the nitrogen wash-out test.²² As to the underlying mechanism, one can only speculate that it may be related to the composition of the surfactant where minimally increased airway surface tension will increase airway closure. Another explanation is that the geometry of the small airways is different in the transplanted lung and/or that loss of alveolar attachments results in increased airway closure or reduced loss of particles during the exhalation. Taken together, these findings signify the presence of ongoing low-grade inflammation in the graft, a factor known to increase the risk of BOS development and possible to ameliorate therapeutically in some patients.²³

An unexpected result was that LTRs had lower concentration of albumin in PEx than controls. Whether this is a true reduction of albumin or rather an increase of another constituent in surfactant of the graft, other proteins or phospholipids, is not known. We have previously shown that the most common phospholipid, dipalmitoyl-phosphatidyl choline, is altered in asthma with an increase in those treated with inhaled glucocorticoids.²⁴

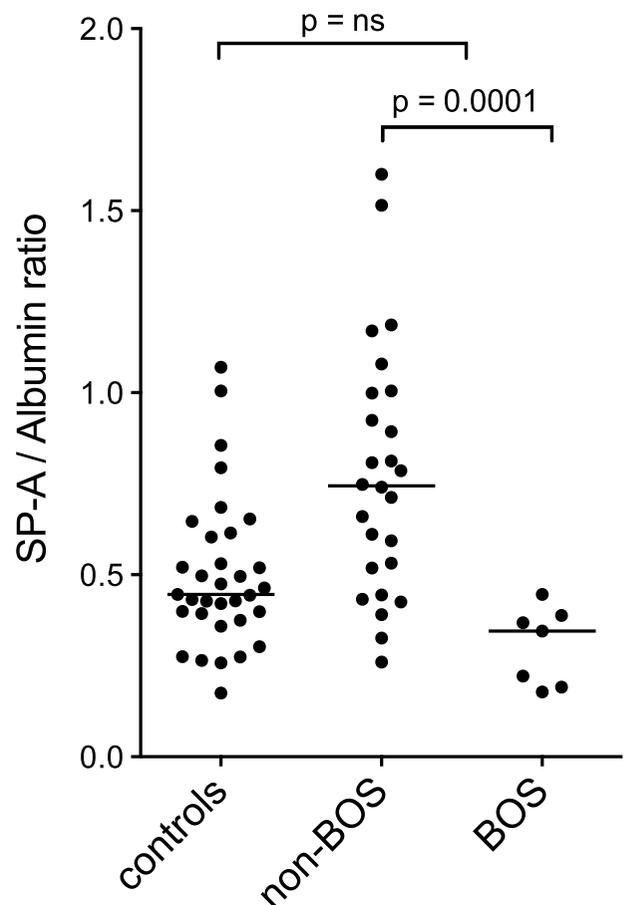


FIGURE 5. The SP-A (mg mL^{-1})/Albumin (mg mL^{-1}) ratio measured by ELISA in PEx from healthy controls ($n = 33$) stable lung transplant recipients (non-BOS) ($n = 26$) and patients that developed BOS ($n = 7$). Bars represent the median.

Seven LTRs received grafts treated with the EVLP method. Ex vivo lung perfusion contributes to expansion of the lung donor pool, thereby decreasing mortality on the transplantation waiting lists. Earlier results demonstrate that the method is safe and enables marginal lung allografts to be recovered and subsequently used for transplantation.²¹ In our study, we found no evidence that grafts treated with EVLP before transplantation differed from “standard” grafts with respect to particle mass concentrations or SP-A and albumin concentrations.

The percentage of single lung transplants was higher in the BOS group. The question whether the remaining nontransplanted lung contributes to the results remains to be answered since the sample size is too small for reliable statistical evaluation.

The PExA instrumentation developed in-house is a novel method that enables sampling from the distal airways without the risk and artifacts associated with bronchoscopy and BAL. It is easy for the patient to perform and has the additional advantage of enabling repeated measurements over time. It would therefore be a considerable improvement in the clinical follow-up of LTRs if the results of this method proved to be clinically robust. However, the power of our study is limited because of its small sample size.

In conclusion, PEx composition differed between stable LTRs and patients that developed BOS with significantly lower SP-A concentrations in the BOS patients. We believe that PExA, a noninvasive diagnostic method that is easy to perform and enables repeated measurements, can facilitate our understanding of the biological processes occurring in the distal airways during allograft rejection and possibly predict BOS onset. However, this is an exploratory study and our results need to be substantiated with longitudinal, prospective data exploring the potential of the PExA method as a useful tool in the follow-up after lung transplantation and SP-A in PEx as a predictive biomarker for BOS.

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