Surfactant protein A and albumin in particles in exhaled air

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Particles exhaled;
Respiratory tract lining fluid;
Surfactant protein A

Summary
In this study we test the hypothesis that endogenous particles in exhaled air (PEx), non-invasively sampled from lower airways, are well suited for the analysis of respiratory tract lining fluid (RTLF) proteins, i.e., surfactant protein A (SP-A) and albumin.

Ten healthy volunteers were included in the study and participated in two sampling sessions. Blood, exhaled breath condensate (EBC) and PEx were collected at each session. 100 L of breath were collected for each exhaled sample. Serum and exhaled samples were analyzed for SP-A using an in-house ELISA. Albumin was analyzed in exhaled samples using a commercial ELISA kit.

SP-A detection rates were 100%, 21%, and 89% for PEx, EBC and serum, respectively. Albumin was detected in PEx, but not in EBC. SP-A and albumin showed significant correlation to mass of PEx (r = 0.93, p < 0.001 and r = 0.86, p = 0.003, respectively).

Sampling and analysis of PEx is a valid non-invasive method to monitor RTLF proteins sampled from the lower respiratory tract, as demonstrated here by example of SP-A and albumin analysis. © 2011 Elsevier Ltd. All rights reserved.

Introduction
Adult humans inhale about 10,000 L of air daily, exposing our respiratory system to air pollutants and pathogens that are potentially hazardous to health. In spite of the relative accessibility of the respiratory system in comparison to other organs, direct assessment of health effects from environmental and occupational exposures is challenging. At present, there are very few methods available for monitoring respiratory diseases affecting distal airways, e.g., chronic obstructive pulmonary disease (COPD) or interstitial lung disease. Standard procedures for collecting

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samples from the respiratory tract are either invasive, such as bronchoalveolar lavage (BAL), or associated with methodological difficulties such as sputum induction and exhaled breath condensate (EBC). At present, EBC analysis is the only commercially-available non-invasive method for collecting samples from the respiratory tract. However, non-volatile material from exhaled air. The method is non-invasive and is based on the collection of endogenous Particles in Exhaled air (PEx). The majority of the sampled particles are likely to be formed during the opening of the distal airway and to follow the airstream during subsequent exhalation. Sampling of PEx includes simultaneous counting of number of exhaled particles in different size-ranges, which is advantageous in quantitative analysis.

The study described in this paper tests the hypothesis that sampling particulate material in exhaled air is a reproducible method for collecting the respiratory tract lining fluid from the distal airways and can be used to quantify surfactant protein A (SP-A) and albumin levels. SP-A and albumin, two proteins known to be abundant in respiratory tract lining fluid (RTLF) from the distal airways, were selected to evaluate correlation between detected protein amounts and the mass of collected PEx. SP-A is the major surfactant protein and is produced locally by alveolar type II cells. SP-A is important for both host defense mechanisms and surfactant function, and is considered to be a potential biomarker for airway inflammation for respiratory diseases, such as asthma. COPD, idiopathic pulmonary fibrosis and bronchiolitis obliterans syndrome. Albumin is the major blood protein and is abundant in RTLF because of the leakage of plasma protein into the airways.

Material and methods

Study population

Ten healthy volunteers from Gothenburg, Sweden, participated in the study; five women and five men. One subject did not perform the breathing maneuver correctly; therefore data obtained from this subject were excluded from the study. The results from the remaining nine subjects are presented. All participants were non-smokers, and underwent a spirometry test (Spirare 3 sps 310, software version 3.35.11, Oslo, Norway) in accordance with ERS guidelines. On the basis of the spirometry tests and their responses to questionnaires, all of the participants were judged to be in good health. Table 1 provides additional information on the subjects.

Study design

Each subject participated in two sampling sessions over the course of one week. In each of these sessions, three types of samples were acquired: EBC, PEx and serum. The sampling sessions took place between 09:00 and 12:00 AM to reduce the influence of diurnal variation. The EBC sample was collected first, followed by sampling of PEx; the same quantity of exhaled air was sampled in both cases. EBC was sampled using tidal breathing, while subjects were requested to perform a predefined breathing maneuver (described in details later in this paper) during the acquisition of PEx samples. The breathing maneuver was chosen on the basis of the results obtained from our previous study on PEx formation. To examine the influence of the breathing pattern on the contents of the EBC, a second sample of EBC was obtained from three of the subjects using the breathing maneuver employed in PEx sampling.

Serum

Blood was drawn into serum gel tubes (Vacuette SST, greiner bio-one GmbH, Kremsmünster, Austria), allowed to clot for 45 min and centrifuged at 1320 × g for 10 min at room temperature. The serum was transferred to 2 ml Protein LoBind Tubes (Eppendorf, Hamburg, Germany) and stored at −20 °C prior to analysis.

PEx

PEx were collected with an instrument developed in-house as described previously. The instrument was modified to control the flow path of the exhaled air, enabling us to sample exclusively exhalations performed with a specific breathing maneuver. Subjects rinsed their mouth with distilled water and breathed filtered air for 2 min before the sampling started. All subjects wore a nose clip throughout the entire procedure. The test subjects were instructed to perform the following three-step breathing maneuver during sampling:

i. Exhale fully until only the residual volume remains in the lungs and hold their breath for a few seconds.

ii. Inhale rapidly until the lungs are filled to their vital capacity.

iii. Exhale back to residual volume at a peak flow of 1000−1500 mL s⁻¹. The exhalation flow was shown to the test subject on a computer screen.

Only iii, the last exhalation was sampled in the instrument. The numbers of particles were monitored using an online particle counter. The number of PEx is defined as the sum of particles with an aerodynamic diameter of \( d = 0.41−4.55 \) µm that are sampled by the impactor. Between breathing maneuvers, the test subject breathed particle-free air tidally. The procedure was repeated until the total volume sampled was 100 L. The sample was extracted from the silicon plate by ultrasonication for 5 min in 115 µL of a desorption solvent consisting of 0.01 M PBS at pH 7.4 (Medi-cago AB, Uppsala, Sweden) containing 0.13% Tween-20 (Bio-
Rad, Hercules, CA, USA) and diluted with 200 μL water. The solution was then transferred to 2 mL Protein LoBind Tubes and dried thoroughly by evaporation under reduced pressure. Samples were stored dry at –20 °C prior to analysis. For the analysis, the dry samples were reconstituted by adding 115 μL MilliQ-water.

**Exhaled breath condensate**

EBC was collected using an ECoScreen instrument (Jaeger, Wurzburg, Germany) and the exhaled volume was measured with an EcoVent flow meter (Jaeger, Wurzburg, Germany). The flow meter was calibrated with a 3 L calibration syringe from New Diagnostic Designs (Zurich, Switzerland) at the start of the study and the calibration was verified at the end of the study. Subjects rinsed their mouth with distilled water before sampling and then breathed tidally while wearing a nose clip, until the total exhaled volume reached 100 L. The lamellar tube was allowed to reach room temperature and then centrifuged for 5 min at 200 × g to ensure that the sample was located in the obtainer at the bottom of the tube. The EBC sample was then transferred from the obtainer to 2 ml Protein LoBind Tubes and stored at –20 °C. Prior to analysis, EBC samples were dried thoroughly by evaporation under reduced pressure and then reconstituted in 115 μL of a solution of 0.01 M PBS at pH 7.4 containing 0.13% Tween-20.

**Albumin ELISA**

Albumin was determined with a commercial E-80AL ELISA kit (Immunology Consultant Laboratory, Newberg, OR, USA) using the protocol suggested by the manufacturer. 10 μL of sample was used and all samples were analyzed using the same ELISA plate. Plate absorbance was read at 450 nm by BioTek ELx-808UI (Highland Park, MI, USA). The concentration of albumin in the samples was calculated from a four parametric standard curve using BioTek software KC Junior V1.41.8 (Highland Park, MI, USA).

**SP-A ELISA**

SP-A was measured using a sandwich ELISA as described by Ellingsen et al. The primary antibody was AB3422 (Millipore, Billerica, MA, USA); the secondary antibody was HYB 238-04 (Antibody Shop, Gentofte, Denmark). 100 μL of sample was used in the SP-A ELISA and all samples including a negative control sample were analyzed on the same ELISA plate. As a reference and standard for a calibration curve, aliquots from pooled serum samples with a known high response. No pure reference protein standard of known amount was available therefore SP-A amount is reported in arbitrary units (au).

**Validation**

The coefficient of variation (CV) for the albumin assay was below 5% as calculated from a single PEx sample, split into four equal parts prior to analysis. The limit of detection (LoD) was estimated to be 3.0 ng mL\(^{-1}\) for albumin on the basis of the lowest standard reported by the ELISA software (recovery 74%, CV 7%).

To validate the inter- and intra-assay CV of the SP-A assay, a pooled PEx sample of 1000 L exhaled breath obtained from six test subjects was split into eight aliquots and analyzed in quadruplicate on two ELISA plates. The observed intra-assay CV was 3%; the inter-assay CV was 25%. Different batches of antibodies were used in the first and second assays.

A negative control was included in every ELISA run. The only difference between the negative control and a sample was that no exhaled breath was sampled when the control was placed in the PEx instrument in all other ways it was treated as a sample. The negative control was placed in the PEx instrument for 30 min as this is approximately the sampling time required. The negative control was below detection limit in both the SP-A and albumin assay.

The loss of material during the extraction procedure was tested using two serum samples with known SP-A concentrations. Samples were spotted onto silicon plates as ten spots of 1 μL each and left to dry for 1 h at room temperature. The sample spots were then frozen for an hour at –20 °C before being extracted and analyzed according to the protocol described previously. The mean recovery for the two samples was 93% (range 4.6–4.7 au).

To evaluate the PEx matrix and possible interference from lipids, serum samples were mixed with Curosurf (Nycomed GmbH, Zurich, Switzerland), a commercially available pig surfactant. Two concentrations of Curosurf were tested (8.0 and 0.8 mg mL\(^{-1}\)). The Curosurf/serum mixture was spotted onto a silicon plate (10 spots of 1 μL, per plate), allowed to dry and then extracted as described previously. Duplicates of three concentrations of Curosurf (0, 0.8, 8.0 mg mL\(^{-1}\)) were analyzed and the coefficient of variation was found to be 8% (mean amount 12.7–10.9–12.2 au respectively). In addition a standard addition test was performed to evaluate matrix effect. A pooled sample obtained from 900 L of exhaled air was extracted and split into five equivalent samples. To these five aliquots, standard was added in increasing concentration to construct a standard curve. The average increase in each spiked sample compared to standard without sample was 0,9 and the slope of the standard curve without sample was not significantly different to that with sample (linear regression 0.208 and 0.208).

To evaluate the storage stability of SP-A 200 L of exhaled breath was sampled as previously described. Half of the sample, seen as five spots on the silicon surface (out of ten spots), was extracted from the plates with extraction solvent (0.01 M PBS/0.13% Tween-20) by pipetting and the extract was analyzed on the same day. The other half was stored for eight months at –20 °C prior to analysis. The storage test was performed twice to get a more reliable result. The recovery after storage was 86% and 93%. The LoD was estimated to be 0.2 au for SP-A (relative error <50% with 95% CI).

**Statistics**

If the concentration of a given analyte in a sample was below the limit of detection, it was assigned to be equal to LoD/\(\sqrt{2}\). When calculating intra-individual coefficient of variation (CV) only test subjects that had two samples above LoD were included. The overall intra-individual
variation was calculated as the mean of the CV for each of the subjects. The inter-individual CV was calculated from the mean concentration for each subject. CV values, means and standard errors were calculated using Microsoft Excel 2007. SAS v9.2 (Raleigh-Durham, NC, USA) was used to calculate Spearman correlation coefficients and quartiles. The significance level was set to $p < 0.05$.

**Ethics**

All participants gave their written informed consent. The study protocol was approved by the Regional Ethical Review Board in Gothenburg.

**Results**

**SP-A**

SP-A amounts from the nine subjects are presented in Table 2. SP-A was detected in all PEx samples, in four EBC samples and in 16 serum samples. There was no significant correlation between PEx-SP-A and serum-SP-A. The intra-individual CV for PEx was 13% and the inter-individual CV was 25%. In serum, the intra-individual CV was 7% and the inter-individual CV was 127%. The relationship between individual means for estimated mass of sampled particles and SP-A is shown in Fig. 1.

**Albumin**

Albumin amounts are presented in Table 3. Albumin was detected in 14 PEx samples and (at a low amount) in one EBC sample. For albumin in PEx samples, the intra-individual CV was 50% and the inter-individual CV was 104%. The relationship between individual means for estimated mass of sampled particles and albumin is shown in Fig. 2.

**Correlation between the mass of PEx and the protein amounts**

The correlation between the amount of SP-A and the mass of PEx was highly significant ($r_s = 0.93, p < 0.001$). There was also a highly significant correlation between the amount of PEx and the amount of albumin ($r_s = 0.86, p = 0.003$).

**EBC sampled using the predefined breathing maneuver**

Three subjects underwent an additional sampling of their EBC (100 L of exhaled air) using the same breathing maneuver as was used when acquiring the PEx samples, i.e. maneuver that induces airway closure and re-opening. In all three samples, the concentrations of SP-A were below the LoD. Only one subject had an albumin amount above the LoD; the albumin amount in this case was 17.2 ng.

**Discussion**

SP-A was readily and repeatedly detected in PEx; the intra-individual CV for SP-A was 13% for two sampling occasions within one week. This stands in contrast to the situation with EBC samples of the same exhaled volume, in which only 4 out of 18 samples exhibited SP-A concentrations above the LoD. Albumin could also be detected in most PEx samples but not in EBC samples. These data indicate that for sampling non-volatile compounds, the PEx method is more efficient than using the EBC approach.

<table>
<thead>
<tr>
<th>Subject</th>
<th>PEx-SP-A (au)</th>
<th>EBC-SP-A (au)</th>
<th>Serum-SP-A (au)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Session 1</td>
<td>Session 2</td>
<td>Session 1</td>
</tr>
<tr>
<td>1</td>
<td>1.4</td>
<td>1.5</td>
<td>&lt;LoD</td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
<td>3.3</td>
<td>&lt;LoD</td>
</tr>
<tr>
<td>3</td>
<td>3.5</td>
<td>2.7</td>
<td>&lt;LoD</td>
</tr>
<tr>
<td>4</td>
<td>3.4</td>
<td>2.8</td>
<td>&lt;LoD</td>
</tr>
<tr>
<td>5</td>
<td>2.2</td>
<td>1.9</td>
<td>1.65</td>
</tr>
<tr>
<td>6</td>
<td>2.5</td>
<td>3.0</td>
<td>0.57</td>
</tr>
<tr>
<td>7</td>
<td>2.1</td>
<td>2.8</td>
<td>&lt;LoD</td>
</tr>
<tr>
<td>8</td>
<td>2.2</td>
<td>3.0</td>
<td>&lt;LoD</td>
</tr>
<tr>
<td>9</td>
<td>2.4</td>
<td>2.2</td>
<td>&lt;LoD</td>
</tr>
<tr>
<td>Median (Q1–Q3)</td>
<td>2.6 (2.2–3.0)</td>
<td>–</td>
<td>15.6 (1.3–17.9)</td>
</tr>
</tbody>
</table>

**Table 2** Amount of sampled surfactant protein A in arbitrary units (au).

PEx-SP-A: The amount of surfactant protein A in sample collected from 100 L using the method based on sampling particles in exhaled air.

EBC-SP-A: The amount of surfactant protein A in sample collected from 100 L of breath using the exhaled breath condensate method.

Serum-SP-A: The amount of surfactant protein A in 100 μL of serum sample.
SP-A and albumin concentrations in PEx were strongly correlated with the mass of PEx. This observation is in agreement with our hypothesis that non-volatile compounds in exhaled air can be transported as aerosol particles. The extent to which these aerosolized particles are formed and exhaled is highly dependent on the nature of the subject’s breathing.\textsuperscript{2,3,16} The breathing maneuver described above, in which the bulk of the PEx are formed by the closure and re-opening of the airways,\textsuperscript{3} provides control over the sample origin. Using this breathing maneuver, RTLF from the terminal bronchioles is the most likely source of the SP-A in PEx collected from healthy subjects.

One of our hypotheses was that the mass of particles in PEx samples should reflect the amount of the collected RTLF. The results demonstrated that this hypothesis is valid for both SP-A and albumin.

### Comparison to existing methods

Like the EBC approach, the PEx method involves sampling of material from exhaled air. However, unlike EBC instruments that use cooling to condense water vapor and in doing so, possibly catching some droplets from the aerosol, the PEx instrument uses a cascade impactor developed for sampling particles. The amount of non-volatile material in exhaled breath may vary significantly among individuals and there is no reason to believe that particle formation is kept constant or is related in a constant fashion to the production of water vapor.\textsuperscript{17} The number of exhaled particles is not monitored in the EBC method, and measured aqueous concentrations cannot be adjusted according to the amount of sampled material. EBC instruments are designed for sampling during tidal

### Table 3 Albumin amount in PEx and EBC and the total mass in sample.

<table>
<thead>
<tr>
<th>Subj</th>
<th>PEx-Albumin (ng)</th>
<th>EBC-Albumin (ng)</th>
<th>ng of PEx ($d = 0.41 - 4.55 \text{ \mu m}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Session 1</td>
<td>Session 2</td>
<td>Session 1</td>
</tr>
<tr>
<td>1</td>
<td>2.65 (0.21–9.60)</td>
<td>—</td>
<td>51</td>
</tr>
<tr>
<td>2</td>
<td>9.60</td>
<td>17.21</td>
<td>315</td>
</tr>
<tr>
<td>3</td>
<td>7.21</td>
<td>9.60</td>
<td>312</td>
</tr>
<tr>
<td>4</td>
<td>12.82</td>
<td>17.26</td>
<td>251</td>
</tr>
<tr>
<td>5</td>
<td>4.42</td>
<td>2.50</td>
<td>99</td>
</tr>
<tr>
<td>6</td>
<td>0.53</td>
<td>9.36</td>
<td>160</td>
</tr>
<tr>
<td>7</td>
<td>&lt;LoD</td>
<td>1.02</td>
<td>72</td>
</tr>
<tr>
<td>8</td>
<td>&lt;LoD</td>
<td>&lt;LoD</td>
<td>&lt;LoD</td>
</tr>
<tr>
<td>9</td>
<td>&lt;LoD</td>
<td>2.80</td>
<td>&lt;LoD</td>
</tr>
<tr>
<td>Median (Q1–Q3)</td>
<td>160.4 (98.5–310.0)</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

PEx-SP-A: The amount of albumin in sample collected from 100 L air using the method based on sampling particles in exhaled air.
EBC-SP-A: The amount of albumin in sample collected from 100 L of air using the exhaled breath condensate method.
PEx is the total mass of sample estimated from total number of particles sampled in the diameter interval $d = 0.41 - 4.55 \text{ \mu m}$.
breathing and tidal breathing produces only a small amount of particulate material compared to the breathing technique used in the PEx method. Condensation in general is not an efficient way to sample particles. The non-volatile material found in ECB is most likely collected by impaction or gravitational settling of aerosol particles in the apparatus. These processes are aided by the growth of the particles due to condensation on cooling. Another possibility is thermophoretic transport toward the walls of the cooler if the thermal gradient is large. This implies that the ECB collection causes size discrimination of the collected material and might explain why particles that contain SP-A and albumin¹⁸ are not efficiently transferred to the EBC sample, and why no improvement was observed when the PEx breathing maneuver was applied in EBC sampling.

It is likely that, PEx are sampled from the distal airways. Therefore, PEx could potentially serve as a non-invasive alternative to BAL for monitoring inflammation in the distal part of the lung. In contrast to BAL sampling, PEx is simple, does not require special medical training on the part of the practitioner or sedation of the subject and is suitable for repeated sampling. Therefore the PEx method has advantages that could make it useful for monitoring the development of respiratory diseases and the progress of their treatment in larger populations. Sputum induction is a less invasive method for sampling RTLF than is BAL, but it is more cumbersome and primarily samples material from the central airways.¹⁹ A key advantage of the PEx method relative to both BAL and sputum induction is that it does not disrupt the RTLF by introducing foreign substances that can induce changes in the RTLF composition or dilute the sample in an unpredictable manner. The limitation of the PEx method compared to BAL and sputum is that no cells are sampled and only low amounts of extracellular material are collected.

We did not observe any correlation between the levels of SP-A in serum and those in PEx, indicating that serum SP-A is not a relevant measure of the concentrations of these proteins in RTLF. The concentrations of SP-A in the serum of the subjects (all of whom were in good health) of this study varied over a wide range (100-fold) between different subjects, but the intra-individual variation was low (7%). This high variability between individuals has been described in a previous study using the same antibody. In healthy individuals it seems unlikely that these differences reflect differences in the alveolo-capillary barrier.

Limitations of the study

The exhalations sampled in the PEx method contain very small amounts of particulate material (approximately 50–400 ng based on particle data). To maximize recovery, the solvent volumes used in processing and extracting the samples were kept to a minimum and the samples were concentrated by vacuum evaporation. Vacuum evaporation could potentially alter the protein structure and the ELISA results. However, we observed good recovery during the validation experiments, suggesting that this is unlikely to be a problem in this study.

In this study we used a predefined breathing maneuver to ensure that the particles exhaled by each individual subject were formed by the same mechanism. Even though there are strong indications that airway opening in the distal airways is the major source of particles when this breathing maneuver is used, the possibility of confounding sources, such as dynamic compression in more central airways still exists. However, no amylase contamination was detected in PEx samples pooled from several thousand liters of exhaled breath using liquid chromatography-mass spectrometry.²⁰

SP-A analyses were limited by the lack of a pure standard, especially since the standard matrix was different from the PEx matrix. All the samples were
treated in the same way; therefore, it was possible to assess their relative SP-A concentrations. We did not test for cross-reactivity in the SP-A ELISA, this has previously been done by Madsen et al\(^4\) who did not detect any cross-reactivity using the same monoclonal antibody (HYB 238-04) in BAL samples. The PEx matrix is less complex than the BAL matrix and since the analyzed proteins are major components of the PEx samples\(^26\), this is likely to further reduce the risk of interfering cross-reactivity for the PEx samples.

**Clinical relevance**

There is a need for better tools to diagnose and monitor respiratory diseases such as asthma and COPD. It is well established that respiratory symptoms only to a minor extent reflect airway inflammation and spirometry is often normal in the early stage disease. To be able to perform repeated measurements and follow the progression of disease new methods are warranted. Today we are lacking the methods for monitoring in the RTLF, particularly in the distal airways. Our data show that the PEx method has potential to become a valuable tool for monitoring inflammatory processes.

**Conclusions**

Sampling and analysis of PEx is a valid non-invasive method for detecting RTLF proteins, as demonstrated in this study by measuring levels of SP-A and albumin. The mass of exhaled particles correlate strongly with the measured concentrations of these proteins, and the results obtained are highly reproducible. Future research in this area will focus on expanding the scope of the PEx method by evaluating it as a tool for examining patients with respiratory disease.

**Conflict of interest**

Two of the authors (A-C Olin and A Almstrand) have a pending patent in Sweden on the method for sampling exhaled particles (P17584SE00). The authors have no other financial interests to declare.

**Acknowledgments**

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