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TOF-SIMS analysis of exhaled particles from patients with asthma and healthy controls

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Abstract

Particles in exhaled air (PEx) may reflect the composition of respiratory tract lining fluid (RTLF) but there is a need for assessing their potential as sources of biomarkers for respiratory diseases**.** In the present study we compared PEx from patients with asthma and controls using Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS) and multivariate analysis.

Particles were collected using an instrument developed in-house. Fifteen non-smoking subjects with physician-diagnosed asthma and 11 non-smoking healthy controls performed 10 consecutive forced exhalations into the instrument. Particle concentrations were recorded and samples of particles collected on silicon plates were analyzed by TOF-SIMS.

Subjects with asthma exhaled significantly lower numbers of particles than controls ($p=0.03$), and the ratio of unsaturated to saturated phospholipids was significantly lower in samples from subjects with asthma (0.25 vs. 0.35, $p=0.036$). Orthogonal partial least squares-discriminant analysis (OPLS-DA) models showed good separation between both positive and negative spectra. Molecular ions from phosphatidylcholine and phosphatidylglycerol and protein fragments were found to discriminate the groups.

We conclude that analysis of PEx is a promising method to examine the composition of RTLF. In the present explorative study, we could discriminate between subjects with asthma and healthy controls based on TOF-SIMS spectra from PEx.

INTRODUCTION

Breath analysis is a non-invasive method of gaining information on the respiratory tract and can be useful for monitoring airway disease. Non-volatile substances in breath are transported in the form of airborne droplets, i.e. particles. Collection of particles in exhaled air (PEx) is a new technique to sample endogenous particles that derive from the respiratory tract. In a previous pilot study we showed that analysis of PEx using Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS) provided substantial information on the phospholipid composition [1]. Only 10 exhalations are required to detect phospholipids and no sample preparation is needed.

Phospholipids are characteristic components of the surfactant that covers the mucous membranes of the airways [2]. The most abundant lipid is dipalmitoylphosphatidylcholine (PC 32:0), which is vital for maintaining a sufficiently low surface tension in the airways. Phosphatidylglycerol (PG), phosphatidylinositol (PI) and sphingomyelin are also present in lower amounts. PG and PI have recently been shown to play important roles in suppressing inflammatory responses [3,4]. The phospholipid composition, as measured in bronchoalveolar lavage (BAL), is altered in various respiratory diseases but the reasons for the changes are uncertain [2]. Thus, analyses of phospholipids in BAL can provide potentially valuable indications of physiological states, but they are far from straightforward since both the sample preparation and analytical methods involved are laborious and the results may be influenced by large variations in sample recovery and dilution. In addition, BAL is an invasive method associated with certain risks for the patient.

In this study, we investigated and compared TOF-SIMS mass spectra and the composition of phospholipids of PEx from subjects with asthma and controls. TOF-SIMS generates comprehensive spectral data and is therefore attracting increasing interest as a tool for classifying biological samples [5-7]. However, since the method is only semi-quantitative and

visual interpretation of spectra is difficult, multivariate analysis of the acquired data is often very useful [7-9]. Thus, multivariate analysis was applied here, using Principal Component Analysis followed by Orthogonal Partial Least Squares (OPLS), to screen TOF-SIMS spectra. We hypothesize that, using this approach TOF-SIMS can be used to discriminate between subjects with asthma and healthy controls and that the phospholipid composition differs between the groups.

METHODS

Study population

Fifteen non-smoking subjects with physician-diagnosed asthma (eight females) and 11 non-smoking healthy controls (six females), free from symptoms of respiratory infection for at least three weeks, participated in the study. They all responded to a respiratory questionnaire based on previous validated items and subjects with asthma were in addition evaluated by a clinical allergist to confirm diagnosis.

Asthma diagnosis was based on the presentation of typical symptoms as defined by GINA guidelines[10]. A significant measure of variable airway obstruction was documented in 14 out of 15 subjects with asthma as indicated by increased PEF-variability (≥ 20 percent variation compared to mean within one week), a positive metacholine challenge test (PC_{20} methacholine <8 mg/ml) or a positive reversibility test (12% or 200 ml in FEV_1 after 400 mg of inhaled salbutamol or spontaneous variation in FEV_1 (12%). One of the subjects with asthma was taking high doses of inhaled glucocorticosteroids and long-acting β2 agonists, had asthma since childhood and was reluctant to taper medication. Ten out of the 15 subjects with asthma were taking inhaled glucocorticosteroids daily and one subject only during pollen season.

Atopy was defined as a positive skin prick test to common inhalant allergens.

Control subjects were all regarding themselves as healthy and did not report any respiratory symptoms or any medication. All performed reversibility-test with a negative outcome.
The forced expiratory vital capacity (FVC) and forced expiratory volume in 1 s (FEV_1) of all participants was determined using a dry-wedge spirometer (Vitalograph®). They performed at least three technically acceptable trials in accordance with ERS guidelines [11], and the largest values for forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1) were registered and compared with predicted values [12]. In addition, the mid-expiratory flow between 25% and 75% of FVC ($MEF_{25-75\%}$) was calculated for all subjects.

The clinical examination started with spirometry. Fraction of exhaled NO (FENO) was measured using a handheld device, NIOX mini (Aerocrine, Sweden). A single breath test at a flow rate of 50 ml/s was performed according to ATS/ERS recommendations [13]. After sampling PEx, skin-prick and reversibility tests were performed. Metacholine challenge test were performed at a separate occasion in those subjects where no other objective measure of variable airway obstruction could be obtained.
The study was approved by the Ethics Committee of Sahlgrenska Academy at the University of Gothenburg.

Collection of PEx and TOF-SIMS analysis

Samples were collected by particle impaction using an instrument developed in-house, as previously described [1]. Participants breathed particle-free air (room air filtered through a HEPA filter) for three minutes before sampling, to avoid contamination from exogenous particles, then performed 10 consecutive forced exhalations, with a target flow of 90% (±10%) of their FEV1, into the device. The flow rate was displayed on a computer screen so

that participants could observe and control their breathing. Particle concentrations were recorded with an optical particle counter (Grimm Model 1.108, Grimm Aerosol Technik GmbH & Co, Ainring, Germany). In addition, PEx were collected on silicon plates that were removed from the instrument immediately after sampling and stored in natural plastic wafer shippers (Fluoroware H22-10, Entegris Inc., Chaska, MN, USA) at −20°C, for no longer than 5 months, until analysis. Sample spots were analyzed with a TOF-SIMS IV instrument (IONTOF, Münster, Germany) as previously described [1]. Positive and negative ion TOF-SIMS spectra were acquired from two randomly selected spots in each sample.

Statistical data analysis

Univariate analysis based on non-parametric (Mann-Whitney) tests was applied to assess the validity of findings of our pilot study and earlier observations, using Statistical Analysis Software 9.1 (SAS Institute Inc., NC, USA).

Multivariate regression analysis

For each TOF-SIMS measurement, the spectra from all pixels were summed into a single spectrum, which was binned to a digital resolution of *m/z* 0.2 and converted to Matlab 4-files using in-house software in Python (Python 2.5.2 with NumPy 1.2.1). Signal intensities of individual ions, *m/z* 20-1000, were normalized according to total intensity in the corresponding full spectrum in Matlab 7.6 (Mathworks, Inc., Natick, MA, USA). SIMCA-P+ 12.0 (Umetrics AB, Umeå, Sweden) was used for multivariate analysis, after mean-centering and Pareto scaling the data [14,15].

Principal Component Analysis (PCA) was used to verify the quality of spectra. Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) was then used to identify differences between patient groups [16,17]

PCA is a statistical tool that is used to examine relationships between multiple variables simultaneously, by using latent correlations among them to reduce the dimensions of the data.

Orthogonal Partial Least-Squares-Discriminant Analysis (OPLS-DA) is a multivariate method that can be used to identify and display differences between patient groups in a single graph (OPLS-DA loading plot) showing all measured variables. The analysis also produces a score plot showing the separation of the groups based on the content of the loading plot. Q2 is a measure of the quality in multivariate models. For OPLS-DA, Q2 is based on cross-validation, a procedure in which fractions of data are systematically excluded. For instance, a model may be calculated using 6/7 of observations (here mass spectra) at a time. A sum of squares is then accumulated for the deviations between the modeled responses (group ratings) and the assigned group (Y). These sum of squares, called Prediction Error Sum of Squares (PRESS), are accumulated for all excluded fractions and compared to the total sum of squares for the variation in Y (SS(Y)) by the formula: Q2=1-PRESS/SS(Y) . A value larger than 0.5 is considered large and indicates that the model provides useful systematic information for the present type of biological data.

RESULTS

Characteristics of subjects and the median amounts of PEx collected from them are presented in Table 1. Subjects with asthma exhaled significantly lower numbers of PEx (of all size classes) than controls (p=0.03). The mass median aerodynamic diameter was the same in both groups, 0.73 μm.

TOF-SIMS analysis

The overall patterns of peaks in spectra from subjects with asthma and controls were similar. Typical positive and negative spectra for a healthy subject are shown in Figure 1 a-b. Both fragments and molecular ions— $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ — of PC were observed. In *positive ion spectra*, the major signals were from sodium, potassium and PC fragment ions in the lower mass region, and from various species of PC in the higher mass range. In *negative*

ion spectra, lower mass-regions were dominated by signals from CN^-, CNO^-, silicon oxides (e.g. SiO_2^-, SiO_3H^-), PO_3^- and the free fatty acids C16:1, C16:0, C18:1 and C18:0. In higher mass ranges, signals from phosphatidic acid (PA), phosphatidylglycerol (PG) and phosphatidyinositol (PI) were detected; see Table 2. Overall, there was high variability in signal intensity among spectra from different individuals. In addition, as observed in our previous study [1], signals from molecular ions were generally stronger in the rim of the spots, probably due to matrix effects.

Multivariate analysis

The PCA analysis of both positive and negative spectra showed that the final dataset does not have any outliers or groupings depending on uncontrolled factors, e.g. sample handling or instrument instability.

For the OPLS-DA analysis, data from samples from subjects with asthma and controls were placed in two different groups to see if the mass spectra support this division. The resulting models showed good separation for both positive and negative ion spectra; see score plots in Figure 2a-b. The corresponding OPLS-DA loading plots (Figure 3 and 4) show the variation in *m/z* information that explains the division in the score plot. In this context, an asthma patient sample with a positive value on the score x-axis corresponds to positive values on the y-axis of the loading plot. Peaks contributing to the separation between groups in positive and negative ion spectra are visible in the loading plots. In summary, in the *low mass range* signals from silicon are stronger among samples from subjects with asthma, while sodium and potassium are stronger among controls. Peaks that are commonly observed in TOF-SIMS spectra, such as phthalates (common contaminants from surrounding air) and fragments from organic compounds appear strong for samples from both subjects with asthma and controls. In *the high mass range* molecular ions from phospholipids (PC 30:0+H, PC 32:0+H and PC 32:0+Na) are strong among samples from subjects with asthma. In negative ion spectra,

silicon signals and typical fragments from protein and phosphate are stronger among samples from subjects with asthma. In the high mass range molecular ions from phospholipids, such as PG 28:0, PG 32:0, PG 34:1 and PG 36:1 are higher among samples from subjects with asthma.

In addition, an OPLS-DA analysis was performed solely on positive spectra of samples from subjects with asthma, which were divided into two groups based on the subjects' intake of inhaled glucocorticosteroids. The resulting model showed a complete separation without overlap between subjects taking glucocorticosteroids and subjects not taking glucocorticosteroids (the corresponding score plot is shown in supplementary information). In the higher mass region, signals from molecular ions PC 32:0 were stronger in samples from subjects taking glucocorticosteroids.

The within-sample (between-spot) repeatability in the score plot was estimated by calculating the coefficient of variation using the standard deviation of scores in the predictive component divided by the mean distance between subjects with asthma and controls. To test the within-individual variability in the OPLS score plot, double or triple samples from three controls and one subject with asthma were included in the original model which then was re-fitted. The coefficient of variation within-sample was 14% and within-individual 17%. For ratios of unsaturated to saturated phospholipids, the coefficient of variation within-samples was 4% and within-individual was 26%.

Study of possible matrix effects

Since the controls exhaled significantly higher amounts of PEx than subjects with asthma, the possible effects of variations in sample amounts on the spectra were assessed by a separate OPLS analysis, using solely spectra from control samples, in which particle concentrations were the Y variables and the TOF-SIMS spectra the X variables. A strong correlation was found between particle concentration and variation in molecular ions of phospholipids

(Q2=0.97). PC 32:0 + H, PC 32:0 + Na and PC 32:0 + K (and in the low mass range Si, K, SiOH and phthalate fragments) were stronger in positive ion spectra of samples with low particle amounts. PG 34:1 and PG 36:1 were the identified phospholipids that dominated in negative ion spectra of samples with low particle amounts.

Univariate analysis

We also tested the validity of findings from our previous pilot study by applying univariate analysis to data acquired in the present study. The results indicated, contrary to previous findings, that ratios of levels of PO_3^- to CN^- , CNO^- and the sum of CN^- and CNO^- in PEx did not significantly differ between the groups.

The ratio of unsaturated to saturated phospholipids was significantly lower in samples from subjects with asthma than in samples from controls (0.25 vs. 0.35, p=0.036), despite high variability within each group (0.13-0.42 and 0.14-0.68, respectively).

DISCUSSION

Sampling and analyzing PEx, which have phospholipid profiles indicating an origin in RTLF [1], offer a novel, non-invasive way of examining the physiological status of subjects' airways. In the present study we discriminate between subjects with asthma and healthy controls using multivariate analysis of TOF-SIMS spectra of PEx. Loading plots from the OPLS analysis show that fragment ions and inorganic ions (present in the low mass range) are important differentiators between the groups. It is difficult to interpret the variation in individual fragment peaks, although their pattern is significant for the division of the groups. Hence, we focus on the molecular ions to address possible reasons for the observed differences in the mass spectra. Major differences in this respect were that spectra of samples from subjects with asthma yielded stronger signals of PC32:0 and several PG species compared to controls.

Alterations in the phospholipid composition in RTLF associated with asthma have been little studied; previous studies of associated changes in the composition of airways surfactant have focused on induced sputum or BAL. It has been speculated that in asthma surfactant dysfunction (due to decreased surface activity) is involved in airway obstruction and inflammation [18-20].Wright et al. observed that the proportion of PC32:0 was decreased in sputum, but not in BAL in patients with asthma [21], while Chang et al. found that PC32:0 correlated with eosinophilic cationic protein in sputum of children with asthma [22]. It has also been observed that children with asthma have higher levels of PC32:0 in sputum compared to control subjects [23]. In atopic asthma, experimental antigen exposure has been shown to induce a reduction in PG in large surfactant aggregates collected from BAL [24]. Leakage of plasma proteins seems to be important for inactivation of surfactant [19,21,25]. It is not possible to identify specific proteins by TOF-SIMS, but CN^- and CNO^- are considered unspecific fragments from proteins and peptides, and according to the loading plots, both CN^- and CNO^- were elevated among subjects with asthma. The ratio of proteins to phospholipids can be estimated by comparing signals from CN^- and CNO^-, and the signal from PO_3^- , which is a strong phospholipid fragment. In the present study we observed no significant difference in this ratio between groups. We also investigated the difference in the ratio of saturated to unsaturated phospholipids between the groups and found that the ratio was significantly elevated among asthmatics. Unsaturated fatty acids are more rapidly oxidized than saturated species, suggesting that an increased ratio of saturated to unsaturated species may reflect the increased oxidative stress known to occur in asthma [26].

In both negative and positive ion spectra, molecular ions from phospholipids were stronger in spectra from subjects with asthma. Indeed, no phospholipid molecular ions were stronger in spectra of samples from controls. This distinction may be due to samples from asthmatic subjects containing smaller amounts of particles. The possible matrix effects caused by

variations in sample amounts were therefore investigated further. Some of the phospholipid molecular peaks were found to correlate to the particle amount. In positive spectra, low particle amount were correlated with stronger signals from PC32:0 while in negative ion spectra, low particle amounts correlated with stronger signals from PG34:1 and PG36:1. However, PG28:0 did not correlate with particle amounts, but is important for distinguishing between controls and subjects with asthma. Samples from two control subjects had extremely high particle concentrations (400000 and 2100000 particles, respectively, see table 1 for median values), and since particle concentration was shown to affect the chemical analysis, the investigated relationships were also modeled without data from these subjects. The Q2 values for the resulting models of both negative and positive ion spectra were improved (0.52 vs. 0.91 for positive spectra and 0.57 vs. 0.64 for negative spectra) and the OPLS loadings for the models for asthma and the model for particle amount were not correlated. The division between groups in the new models was stronger than in the original models; see supplemental information for score plots. Furthermore, PC 32:0, PG 32:0, PG 34:1 and PG36:1 were stronger differentiators of the groups in the new models, indicating that the method could be further improved by standardizing sampled particle numbers for TOF-SIMS analysis.

The effect of inhaled glucocorticosteroids on spectra from subjects with asthma was also examined using OPLS-DA. The results indicate that inhaled glucocortisteroids may alter the surfactant composition, and hence the spectra of PEx. . The results agree with studies showing that glucocorticosteroids stimulate the synthesis of PC [27,28]. An alternative explanation is that those taking inhaled glucocortisteroids have a more severe disease and that this is reflected in the mass spectra. The groups were however too small to draw any further conclusions.

It is not known if the number of PEx is affected in airway inflammation. In the present study, controls exhaled significantly higher numbers of PEx than subjects with asthma. Subjects

performed forced exhalations since this maneuver produces much higher particle numbers than tidal exhalations. The subjects were instructed to exhale at a maximal flow corresponding to 90% of their FEV1 (± 10%). The reasoning behind this was that the intra-thoracical flow may be highly important for the formation of particles during forced exhalations, especially during dynamic compression of the airways, which may be an important mechanism for endogenous particle production. In this small group, inhaled glucocorticosteroids did not affect particle number. We have recently shown that airway re-opening is an important mechanism for PEx formation [29]. We have not controlled for this in the present study. However, all subjects were given similar instructions – to inhale as deeply as possible and then exhale maximally. Subjects with asthma may however have been unable to exhale as deep as control subjects because of premature airway closure. The site of airway closure may have taken place at earlier generations of the bronchial tree for subjects with asthma compared to controls which could affect the particle number and possibly also the composition of particles. Furthermore, the location of the flow-limiting segment may differ between subjects with asthma and controls which in turn may also affect the location of particle production if particles are formed due to shear forces produced by the high flows. Differences in the composition of the RTLF may also contribute to the observed difference in PEx concentrations between controls and subjects with asthma. Nebulised isotonic saline has been shown to influence the number of PEx during tidal breathing [30], possibly by influencing the viscosity of the respiratory tract lining fluid. Whether PEx concentrations are really lowered in asthma is a matter for future studies.

Asthma is a very heterogeneous disease, ideally more subjects would have been included and possibly steroid-naïve subjects with a more uniform disease. The subjects with asthma in the present study were recruited by advertisements in the hospital or the university and presented with varying disease severity (from mild-intermittent to moderate) and medication varied.

Hence the present work should be considered an explorative study of both a new sampling method and an analytical method that has not previously been used in respiratory research. We were nevertheless able to show that it is possible to distinguish between subjects with asthma and controls based on TOF-SIMS spectra of PEx and that differences in the phospholipid composition were important for the separation between groups.

CONCLUSIONS

Collection and analysis of PEx seem to be a promising new method for examining the respiratory tract lining fluid and monitoring changes in the composition of surfactant in respiratory disease. The signals from PC32:0 and several species of PG were higher in samples from asthmatic subjects and they also had higher ratios of saturated to unsaturated phospholipids compared to controls.

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Table 1 Basic data on subjects with asthma and controls. Means (ranges) are presented for age, FVC (forced vital capacity), FEV1 (% pred) (percentage of the predicted value), MEF25%-75% (mid-expiratory flow between 25% and 75% of FVC), and FENO. Medians (ranges) are also presented for number of collected particles.

	Controls **n=11**	**Subjects with asthma** **n=15**
Sex (m/f)	5/6	7/8
Age (yr)	37 (21-55)	30 (19-55)
Atopy (n %)	36	79*
FVC, % pred (range)	115.3(93-129)	102.6 (77-126)
FEV1 (% pred)	108 (80-126)	97 (75-116)
$MEF_{25\%-75\%}$	3.8 (1.6-6.0)	3.6 (1.5-6.2)
FENO (ppb)	20 (11-33)	55 (13-223)
Number of collected particles (0.50-2.0 µm)	44 000 (4500-2 100 000)	23 000 (8100-97 000)

*Number based on 14 subjects, data are missing for one subject.

Table 2 Summary of peak assignments of TOF-SIMS spectra obtained from exhaled particles. All *m/z* values correspond to singly charged monoisotopic ions. Molecular species of phospholipids are named *x:a*, where *x* is the number of carbons and *a* is the number of double bonds. Secondary ions are detected as protonated ions $[M+H]^+$, sodiated ions $[M+Na]^+$ and potassiated ions $[M+K]^+$ in positive ionization mode and deprotonated $[M-H]^-$ in negative ionization mode.

Positive ions		Negative ions	
assignment	***m/z***	**assignment**	***m/z***
PC fragment	58.1	C16:1	253.2
PC fragment	86.1	C16:0	255.2
PC fragment	184.1	C18:1	281.2
PC fragment	476.4	C18:0	283.3
PC fragment	478.4	PA 32:1	645.4
Lyso-PC/PC fragment	494.3	PA 32:0	647.4
Lyso-PC/PC fragment	522.3	PG 28:1	663.4
Lyso-PC/PC fragment	524.4	PG 28:0	665.4
PC 28:0+H	678.5	PG 32:0	671.5
PC 30:0+H	706.6	PA 34:1	673.5
PC 30:0+Na	728.5	PG 32:0	721.5
PC 32:1+H	732.6	PG 34:1	747.5
PC 32:0+H	734.5	PG 36:2	773.5
PC 30:0+K	744.5	PG 36:1	775.5

PC 32:1+Na	754.5	PI 34:2	833.6
PC 32:0+Na	756.5	PI 34:1	835.6
PC 34:1+H	760.6	PI 36:2	861.6
PC 34:0+H	762.6	PI 36:1	863.6
PC 32:1+K	770.6		
PC 32:0+K	772.5		
PC 34:1+Na	782.5		
PC 34:0+Na	784.5		
PC 34:1+K	798.6		
PC 34:0+K	800.6		

Figure legends.

Figure 1a A typical positive ion TOF-SIMS mass spectrum from a sample spot of exhaled particles from a healthy control.

Figure 1b A typical negative ion TOF-SIMS mass spectrum from a sample spot of exhaled particles from a healthy control.

Figure 1a

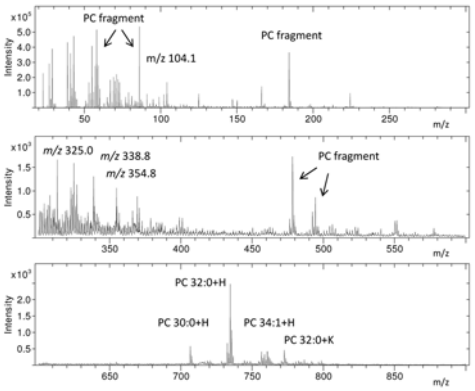


Figure 1b

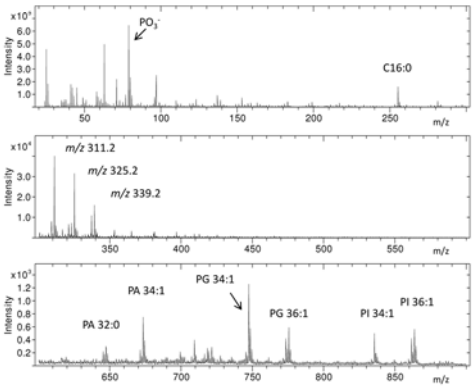


Figure 2a OPLS-DA score plot of positive ion spectra. ■=subjects with asthma ●=control. R2X(cum)=0.606, R2Y(cum)=0.655, Q2(cum)=0.517

Figure 2b OPLS-DA score plot of negative ion spectra. ■=subjects with asthma ●=control. R2X(cum)=0.694, R2Y(cum)=0.602, Q2(cum)=0.568

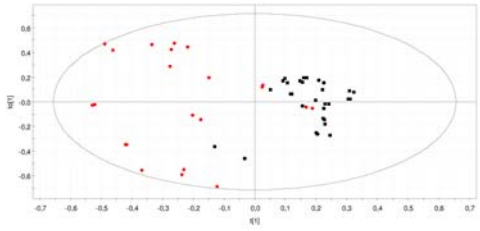


Figure 2a

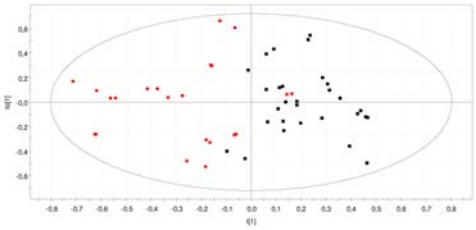
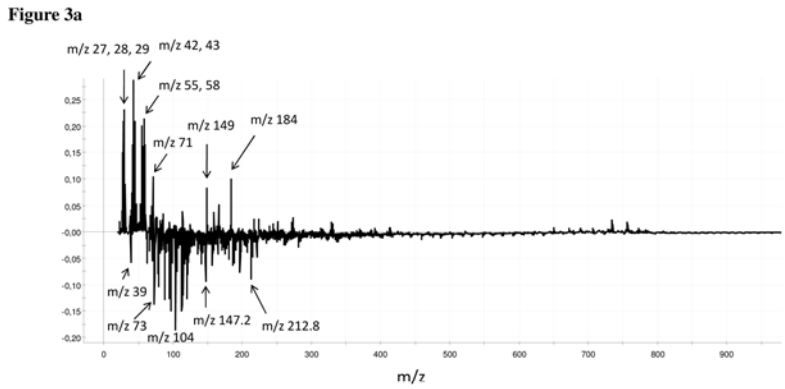


Figure 2b

Figure 3a OPLS-DA loading plot of positive ion TOF-SIMS spectra. Arrows indicate the *m/z* peaks with strong impacts on the separation between groups. A positive value on the y-axis corresponds to *m/z* peaks that are relatively stronger in spectra of samples from subjects with asthma than in control spectra.

Figure 3b Detailed view of the high mass range of the OPLS-DA loading plot (shown in entirety in Figure 3a) of positive ion TOF-SIMS spectra. Identified peaks are indicated.

Figure 4a OPLS-DA loading plot of TOF-SIMS negative ion spectra. Arrows indicate the *m/z* peaks with strong impacts on the separation between groups. A positive value on the y-axis corresponds to *m/z* peaks that are relatively stronger in spectra of samples from subjects with asthma than in control spectra.



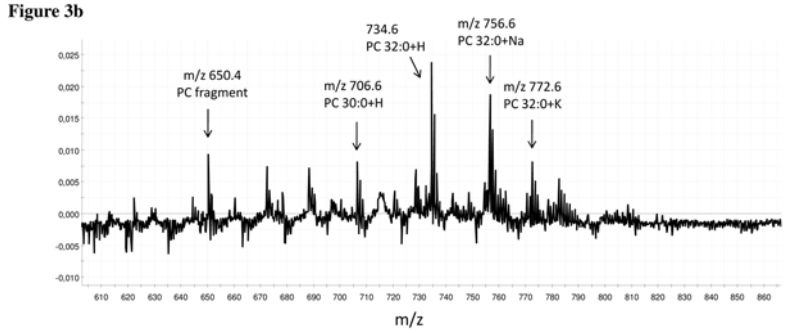
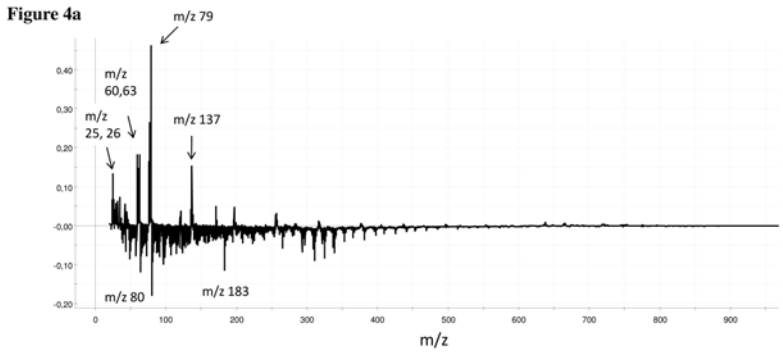
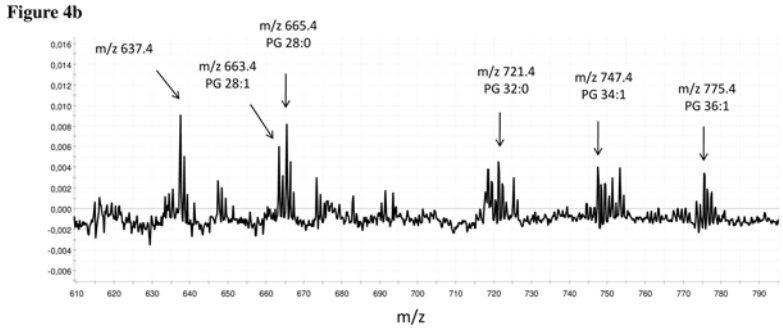


Figure 4b Detailed view of the high mass range of the OPLS-DA loading plot (shown in entirety in Figure 4a) of TOF-SIMS negative ion spectra. Identified peaks are indicated.





REFERENCES

1 Almstrand AC, Ljungstrom E, Lausmaa J, Bake B, Sjovall P, Olin AC: Airway monitoring by collection and mass spectrometric analysis of exhaled particles. Anal Chem 2009;81:662-668.

2 Griese M: Pulmonary surfactant in health and human lung diseases: State of the art. Eur Respir J 1999;13:1455-1476.

3 Kuronuma K, Mitsuzawa H, Takeda K, Nishitani C, Chan ED, Kuroki Y, Nakamura M, Voelker DR: Anionic pulmonary surfactant phospholipids inhibit inflammatory responses from alveolar macrophages and u937 cells by binding the lipopolysaccharide-interacting proteins cd14 and md-2. J Biol Chem 2009;284:25488-25500.

4 Numata M, Chu HW, Dakhama A, Voelker DR: Pulmonary surfactant phosphatidylglycerol inhibits respiratory syncytial virus-induced inflammation and infection. Proc Natl Acad Sci U S A;107:320-325.

5 Sjovall P, Lausmaa J, Johansson B: Mass spectrometric imaging of lipids in brain tissue. Anal Chem 2004;76:4271-4278.

6 Brunelle A, Laprevote O: Lipid imaging with cluster time-of-flight secondary ion mass spectrometry. Anal Bioanal Chem 2009;393:31-35.

7 Wu L, Lu X, Kulp KS, Knize MG, Berman ESF, Nelson EJ, Felton JS, Wu KJJ: Imaging and differentiation of mouse embryo tissues by tof-sims. International Journal of Mass Spectrometry 2007;260:137-145.

8 Park J-W, Min H, Kim Y-P, Kyong Shon H, Kim J, Moon DW, Lee TG: Multivariate analysis of tof-sims data for biological applications.

9 Lhoest JB, Wagner MS, Tidwell CD, Castner DG: Characterization of adsorbed protein films by time of flight secondary ion mass spectrometry. J Biomed Mater Res 2001;57:432-440.

10 GINA: Pocket guide for asthma management and prevention. www.ginasthma.org, Global Initiative for Asthma, 2010,

11 Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, Crapo R, Enright P, van der Grinten CP, Gustafsson P, Jensen R, Johnson DC, MacIntyre N, McKay R, Navajas D, Pedersen OF, Pellegrino R, Viegi G, Wanger J: Standardisation of spirometry. Eur Respir J 2005;26:319-338.

12 Quanjer PH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault JC: 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.

13 American Thoracic S, European Respiratory S: Ats/ers recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. Am J Respir Crit Care Med 2005;171:912-930.

14 Trygg J, Holmes E, Lundstedt T: Chemometrics in metabonomics. J Proteome Res 2007;6:469-479.

15 van den Berge M, ten Hacken NHT, Cohen J, Douma WR, Postma DS: Small airway disease in asthma and copd. Chest 2011;139:412-423.

16 Trygg J, Wold S: Orthogonal projections to latent structures (o-pls). Journal of Chemometrics 2002;16:119-128.

17 Wold S, Trygg J, Berglund A, Antti H: Some recent developments in pls modeling. Chemometrics and Intelligent Laboratory Systems 2001;58:131-150.

18 Kurashima K, Fujimura M, Matsuda T, Kobayashi T: Surface activity of sputum from acute asthmatic patients. Am J Respir Crit Care Med 1997;155:1254-1259.

19 Hohlfeld JM, Ahlf K, Enhorning G, Balke K, Erpenbeck VJ, Petschallies J, Hoymann HG, Fabel H, Krug N: Dysfunction of pulmonary surfactant in asthmatics after segmental allergen challenge. Am J Respir Crit Care Med 1999;159:1803-1809.
20 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.
21 Wright SM, Hockey PM, Enhorning G, Strong P, Reid KB, Holgate ST, Djukanovic R, Postle AD: Altered airway surfactant phospholipid composition and reduced lung function in asthma. J Appl Physiol 2000;89:1283-1292.
22 Chang AB, Gibson PG, Masters IB, Dash P, Hills BA: The relationship between inflammation and dipalmitoyl phosphatidycholine in induced sputum of children with asthma. J Asthma 2003;40:63-70.
23 Shaheen MA, Mahmoud MA, Abdel Aziz MM, El Morsy HI, Abdel Khalik KA: Sputum dipalmitoylphosphatidylcholine level as a novel airway inflammatory marker in asthmatic children. Clin Respir J 2009;3:95-101.
24 Hite RD, Seeds MC, Bowton DL, Grier BL, Safta AM, Balkrishnan R, Waite BM, Bass DA: Surfactant phospholipid changes after antigen challenge: A role for phosphatidylglycerol in dysfunction. Am J Physiol Lung Cell Mol Physiol 2005;288:L610-617.
25 Heeley EL, Hohlfeld JM, Krug N, Postle AD: Phospholipid molecular species of bronchoalveolar lavage fluid after local allergen challenge in asthma. Am J Physiol Lung Cell Mol Physiol 2000;278:L305-311.
26 Ochs-Balcom HM, Grant BJ, Muti P, Sempos CT, Freudenheim JL, Browne RW, McCann SE, Trevisan M, Cassano PA, Iacoviello L, Schunemann HJ: Antioxidants, oxidative stress, and pulmonary function in individuals diagnosed with asthma or copd. Eur J Clin Nutr 2006;60:991-999.
27 Post M, Batenburg JJ, van Golde LM: Effects of cortisol and thyroxine on phosphatidylcholine and phosphatidylglycerol synthesis by adult rat lung alveolar type ii cells in primary culture. Biochim Biophys Acta 1980;618:308-317.
28 Bunt JE, Carnielli VP, Janssen DJ, Wattimena JL, Hop WC, Sauer PJ, Zimmermann LJ: Treatment with exogenous surfactant stimulates endogenous surfactant synthesis in premature infants with respiratory distress syndrome. Crit Care Med 2000;28:3383-3388.
29 Almstrand AC, Bake B, Ljungstrom E, Larsson P, Bredberg A, Mirgorodskaya E, Olin AC: Effect of airway opening on production of exhaled particles. J Appl Physiol 2010;108:584-588.
30 Edwards DA, Man JC, Brand P, Katstra JP, Sommerer K, Stone HA, Nardell E, Scheuch G: Inhaling to mitigate exhaled bioaerosols. Proc Natl Acad Sci U S A 2004;101:17383-17388.