Particles in exhaled Air (PExA): Non-Invasive Phenotyping of Small Airways Disease in Adult Asthma

Marcia Soares, BSc[1]

Ekaterina Mirgorodskaya, PhD[2]

Hatice Koca, BSc, MS [2]

Emilia Viklund, BSc[2]

Matthew Richardson, PhD[1]

Per Gustafsson, MD, PhD[3]

*Anna-Carin Olin, MD, PhD[2]

*Salman Siddiqui, MD, PhD[1]

[1] NIHR Biomedical Research Centre: Respiratory Theme and Department of Infection, Immunity and Inflammation, University of Leicester, United Kingdom.

[2] Occupational and Environmental Medicine, Department of Public Health and Community Medicine, Institute of medicine, Sahlgrenska Academy, University of Gothenburg.

[3] Department of Paediatric Clinical Physiology, Queen Silvia Children's Hospital, S-416 85 Gothenburg, Sweden

*These authors contributed equally to the work.

Corresponding author:

Professor Salman Siddiqui, NIHR Respiratory Biomedical Research Centre and Department of Infection, Immunity and Inflammation, Glenfield Hospital, LeicesterLE3 9QP.

Telephone: +44(0)116 256 3841, email: ss338@le.ac.uk

Author contributions:

MS: Jointly with SS designed the study, recruited the study population, performed PExA collection and analysis, performed and analysed impulse oscillometry and clinical data and conducted statistical analyses. Co-wrote the manuscript with SS.

EM: Designed, coordinated and performed PExA immune assay experiments. Reviewed the manuscript and provided a scientific critique of the data.

HK: Performed the immune assays of PExA for the replication population. Reviewed the manuscript and provided a scientific critique of the data.

EV: Provided data for the PExA asthma replicate population. Reviewed the manuscript and provided a scientific critique of the data.

MR: Overviewed statistical methodologies in the paper. Reviewed the manuscript and provided a scientific critique of the data.

PG: Provided data for the PExA asthma replicate population. Reviewed the manuscript and provided a scientific critique of the data.

AO: Helped conceive the study with SS, Provided the PExA instrument to SS, coordinated analyses of PExA samples by MS, HK & EM at the University of Gothenburg. Reviewed the manuscript and provided a scientific critique of the data.

SS: Conceived and designed the study and obtained funding for the study. Co-wrote the manuscript, reviewed and provided a scientific critique of the data.

Word count: 3472

Key words: Small airways disease, PExA, Asthma, non-invasive technique, physiological tools.

Abbreviations:

ACQ: Asthma control Questionnaire

AQLQ: Asthma Quality of Life Questionnaire

AX: Area of Reactance

BD: Bronchodilator

BMI: Body Mass Index

BOS: Bronchiolitis obliterans syndrome

FEV_1: Forced Expiratory Volume in the first second

FVC: Forced Vital Capacity

GINA: Global Initiative for Asthma

IOS: Impulse Oscillometry

LCI: Lung Clearance Index

MBW: Multiple Breath Washout

OS: online supplement

PBS: Phosphate-Buffered Saline

PC_{20}: Methacholine Challenge test derived provocation concentration causing a 20% decrease in FEV_1 from baseline

PExA: Particles in Exhaled Air method

PEx: Particles in Exhaled Air

Sacin: Acinar Ventilation Heterogeneity

Scond: Conductive Ventilation Heterogeneity

SPA: Surfactant protein A

This article has an online data supplement, which is accessible from this issue's table of content online.

Abstract *(200 words)*

Rationale: Asthma is often characterised by inflammation, damage and dysfunction of the small airways, but no standardised biomarkers are available.

Objectives: Using a novel approach -Particles in Exhaled Air (PExA)- we sought to a) sample and analyse abundant protein biomarkers: surfactant protein A (SPA) and albumin in adult asthmatic and healthy patients and b) relate protein concentrations with physiological markers using phenotyping.

Methods: 83 adult asthmatics and 21 healthy volunteers were recruited from a discovery cohort in Leicester, UK, and 32 adult asthmatics as replication cohort from Sweden. Markers of airways closure/small airways dysfunction were evaluated using forced vital capacity (FVC), impulse oscillometry (IOS) and multiple breath washout. SPA/albumin from PEx (PExA sample) were analysed using ELISA and corrected for acquired particle mass. Topological data analysis (TDA) was applied to small airway physiology and PExA protein data to identify phenotypes. **Results:** PExA manoeuvres were feasible, including severe asthmatic subjects. TDA identified a clinically important phenotype of asthmatic patients with multiple physiological markers of peripheral airway dysfunction, and significantly lower levels of both SPA and albumin.

Conclusion: We report that the PExA method is feasible across the spectrum of asthma severity and could be used to identify small airway disease phenotypes.

Introduction

Asthma is a disease associated with inflammation, remodelling and dysfunction [1- 2] that extends to the smaller airways [3]. However, a standardised definition of small airways disease has remained problematic due to a lack of validated non-invasive physiological and pathological tools to measure dysfunction and disease within this compartment. The existing approaches are either invasive (not suitable for population studies e.g. trans bronchial and surgical lung biopsy) [4] or both invasive and difficult to standardise, such as broncho-alveolar lavage sampling [5].

In the past few years a novel non-invasive technique sampling endogenous aerosol particles, carrying non-volatile material from the small airways (PExA) has been developed, first reported by Almstrand *et al* [6]. The approach has potential advantages, including the ability to sample small airway surface liquid non-invasively and the ability to normalise biomarker concentrations to particle mass. To date, promising but exploratory studies have been conducted using the PExA technique, applying proteomics [7,8,9,10] and lipid analysis of phosphatidylcholines [11-12] that support a small airway organ for PEx particles. Specifically, exhaled particle numbers have been shown to be correlated with physiological measures of airways closure [13] and are most enriched during repeated airways closure and reopening breathing manoeuvres in contrast to tidal breathing [14]. This manoeuvre is therefore likely to promote sampling from the terminal bronchioles, and alveoli. Furthermore, Larsson *et al* have reported that different breathing manoeuvres generate distinct phospholipid profiles and that the airway closure manoeuvre was the most likely manoeuvre associated with the peripheral compartment [15]. Additionally a pilot study in a population with asthma, showing that increased airway inflammation due to birch pollen exposure was associated with less exhaled particles and therefore, suggestive of a reduced number of small airways available for the closure and re-opening and particle generation [8]. Finally, a recent study has

identified ingested methadone in PExA particles, further suggesting a communicative link between PExA particle and the systemic circulation/small airways [16].

These observations would suggest that PEx (PExA sample) may be an appropriate matrix to study asthmatic small airways biology if further validated.

Immunoglobulins, albumin, as well as lung specific proteins such as surfactant proteins have been detected in PEx and are amongst the most abundant proteins [7]. Both albumin and surfactant protein A (SPA) can be measured in PEx at low limits of detection using immunoassays [9-10]. SPA is a key immunomodulatory protein involved in innate immune recognition and regulation of surface tension [17]. For example, SPA can inhibit allergen-specific IgE binding to mite allergens [18], increases phagocytosis of bacteria and viruses by macrophages, monocytes and neutrophils [19]. Previous studies in chronic bronchitis have suggested that surfactant dysfunction may be responsible for airflow obstruction and potentially reversible [20].

Albumin is a plasma protein and its presence in PEx, although not fully understood, may reflect breakdown of the alveolar basement membrane and/or increased airway vascular permeability [21].

Previous studies have evaluated pooled PEx samples for SPA and albumin % in both mild asthma and obstructive lung diseases (COPD and bronchiolitis obliterans syndrome-BOS) [12, 14, 22]. These studies suggest that SPA is associated with obstructive lung disease COPD and BOS, providing a sound justification to study the role of SPA and albumin in asthma and their association with airways dysfunction.

In the present study, we hypothesised that: a) PExA method is both feasible across the spectrum of asthma severity and reliable with respect to quantification of both SPA and

albumin and b) candidate biomarkers SPA and albumin derived from PEx, are associated with both airways closure and small airway dysfunction phenotypes in adult asthmatics.

We sought to test these hypotheses in a discovery and replication cohort.

Methods

Discovery Cohort

The discovery study protocol was approved by the National Research Ethics Committee – East Midlands Leicester (approval number: 08/H0406/189) and all subjects gave their written informed consent.

104 volunteers were screened and recruited at Glenfield Hospital, Leicester, from secondary care asthma clinics, and from an existing research database at the NIHR Respiratory Biomedical Research Centre, Leicester, UK.

83 asthma patients, of which 74 could produce PEx samples with enough material to analyse [Global Initiative for Asthma (GINA) (1), treatment steps: I=9, II-III=25 and IV-V=40] and 21 healthy aged matched volunteers, non or ex-smokers were identified and met the entry criteria of the study outlined below [see **Figure E1** and **Table E1**, online supplement (OS)].

Asthma patients had a physician diagnosis of asthma and one or more of the following physiological criterion: Methacholine PC20 ≤8mg/ml, bronchodilator reversibility to 400 mcg of inhaled salbutamol of FEV_1 ≥12% and 200mls or peak flow variability of ≥ 20% over two weeks. Patients had been free from exacerbations for at least six weeks prior to study entry. Asthma patients currently smoking or with a smoking pack history greater than or equal to 15 were excluded.

Replication Cohort

An additional Swedish cohort of n= 32 asthmatic patients (GINA I-III=18, GINA IV= 14) was evaluated as a replication population (**Table E 6,** OS). Patients were between 27 and 60 years of age, 15 subjects were male, and all had persistent asthma symptoms and were either never or ex-smokers (mean [range] pack year exposure of 7.5 [1.25-16.13]). Patients were recruited from primary care centres across Skaraborg Country in West Sweden, with approval by the Regional Ethics Committee at the University of Gothenburg, Sweden. All patients met the inclusion criteria, as previously reported [23].

Study design

Patients attended for up to two visits within a week. Data obtained during visit 1 were: medical history and current medication, skin prick testing, Juniper Asthma Control Questionnaire [ACQ-6] [24] and Asthma Quality of Life questionnaire [AQLQ] [25] for the asthma cohort, and spirometry plus reversibility. At the second visit, lung physiology measurements were performed 15 minutes following the administration of 400 mcg of Salbutamol via a spacer: impulse oscillometry (IOS), multiple breath washout (MBW), spirometry and PExA.

Induced sputum was performed after PExA, using a previously published standard operating procedure [26]. Samples were sent for differential cell count. Only 42% of patients were able to produce a viable sputum sample, therefore, not included in the main results (see online supplement, **table E3** and **E4**).

212 Physiological measurements

213 Spirometry was performed according to ATS/ERS recommendations [27] using a Vitalograph
214 Alpha AL 21523 device (Vitalograph, Maids Moreton, Buckingham, MK18 1SW) in the
215 discovery cohort and a Jaeger Masterscreen (Care Fusion, Germany) device in the replication
216 cohort.

217 IOS measurements and quality control were performed in line with ERS Task Force
218 recommendations [28] using the Masterscreen IOS (Erich Jaeger/Care Fusion, Germany), in
219 both cohorts. The device was calibrated daily using a standardised resistance and
220 measurements were performed in triplicate, as described previously [29].

221 MBW testing was performed in the discovery population, using a modified photoacoustic
222 INNOCOR (Innovision, Odense, Denmark) SF-6 gas analyser. Measurements were performed
223 2-3 times within visit to ensure that at least two FRC values were within 10% of each other.
224 Several parameters were derived from the raw MBW data using a custom MATLAB software
225 [MATLAB 2015a, Natick, Massachusetts: The MathWorks Inc., 2015], including lung
226 clearance index (LCI) and phase three slope derived measures of conductive (Scond) and
227 acinar (Sacin) ventilation heterogeneity as previously described [30- 31].
228

229 PEx was collected using identical instruments in both cohorts and patients followed the
230 standard breathing manoeuvre [8]: full exhalation till residual volume (RV), followed by
231 breath hold for about 5 seconds and rapid inspiration till total lung capacity, finishing with a
232 steady exhalation back to RV, at a peak flow of about 1500 ml/s **(Figure E2**,OS**)**. Expiratory
233 flow content was split between a Grimm 1.108 optical particle counter (Grimm Aerosol
234 Technik GmbH & Co, Ainring, Germany) to monitor the number of particles collected in each
235 breath.

PEx was extracted using PBS/0.05% Tween and particles SPA/Albumin evaluated using the Human Surfactant Protein A ELISA (BioVendor, Heidelberg, Germany), with monclonal anti-human SPA [32] and a high sensitivity ELISA for Albumin (ICL, Portland, USA) [33]. Further details can be seen on the online supplement (**Figure E3, E4 and E5, table E5**). Total SPA and albumin concentrations were derived from four parameter fitted standard curves and were normalised to acquired PEx mass (ng) to yield a % of SPA and albumin. Intra and inter assay coefficient of variation were < 10% for both SPA and albumin (OS).

Statistical analysis

Statistical analyses were performed using SPSS 22 (IBM Corporation, Somers, NY, USA) and Prism 7 for graphical plots (GraphPad Software Inc., La Jolla, CA, USA). A *p*-value of <0.05 was taken as the threshold for statistical significance. Comparisons between or across groups were performed using either ANOVA/Kruskal–Wallis test for parametric/non-parametric data and the fisher's exact test for proportions. Bonferroni/Dunn corrections for multiple comparisons were used as appropriate. Correlations between continuous variables were calculated using Spearman's correlation coefficient *(Rs*).

Topological Data Analysis (TDA) was utilised to evaluate putative small airway phenotypes in the overall discovery population. The central idea in TDA is that the shape of the data has meaning; by understanding the underlying shape of a data set it is possible to discover interesting features such as clusters or subgroups [34-37]. TDA generates two dimensional networks of nodes connected by lines and edges to neighbouring nodes based upon patient similarity. Nodes in the network represent clusters of patients and edges connect nodes that contain patients that share phenotypic similarity. Nodes are subsequently coloured by the average value of their respective patients for the variables.

TDA offers the advantage of being sensitive to large and small scale patterns, which are often not detected with other methods, such as principal components analysis and cluster analysis. Furthermore, simple cluster analysis requires pre-specification of the number of clusters to be generated and doesn't allow within cluster analysis of clinical heterogeneity; on the other hand, TDA allows for analysis of heterogeneity within a network.

TDA was performed using the Ayasdi Workbench v7.1.0 software (Ayasdi, Palo Alto, California). The construction of networks is based in different input variables, which then allow the identification of networks and sub-groups of interest. In our analysis, the column set comprised Sacin, SPA % PEx, Alb % PEx and R5-R20. The TDA input parameters were chosen with a view to identifying composite phenotypes of both small airways dysfunction and PExA protein associated disease (GINA step treatment, R5-R20, % SPA, % albumin). The TDA metric used was the norm correlation, which is the Pearson correlation coefficient applied to the normalised variables, i.e., variables were transformed to follow a standard normal distribution. The resolution (percent overlap) used was 30, gain 3 (degree of overlap) and the lens applied (based on distance between points in the dataset) were the neighbourhood 1 and 2 lens. Further methodology on the TDA analysis details are described on the supplementary material.

Results

Feasibility Study

PExA repeated airway closure manoeuvres was feasible across the spectrum of asthma severity (**Table E2A, B,** OS), after simple explanation and demonstration.

The median time to collect 50-100 ng of PEx material was approximately 9 minutes (range 5-14) and equated to approximately 4 (2-7) airway closure manoeuvres (**Figure 1A and 1B**). Seven out of 47 patients with severe asthma (GINA IV-V), generated low amounts of particles for biomarkers analysis compared to 2/36 GINA I-III asthmatics (chi squared p value =0.157).

A significant relationship was found between mean number of particles generated per exhalation manoeuvre and airflow obstruction measured with spirometry (FEV_1/FVC) - **Figure 1C**- but not with the protein concentration normalised to ng of acquired PEx mass and (**Figure 1D**), suggesting that the normalisation to ng of mass removed any sampling bias due to airflow obstruction and the number of subtended airways available in the lung for particle generation.

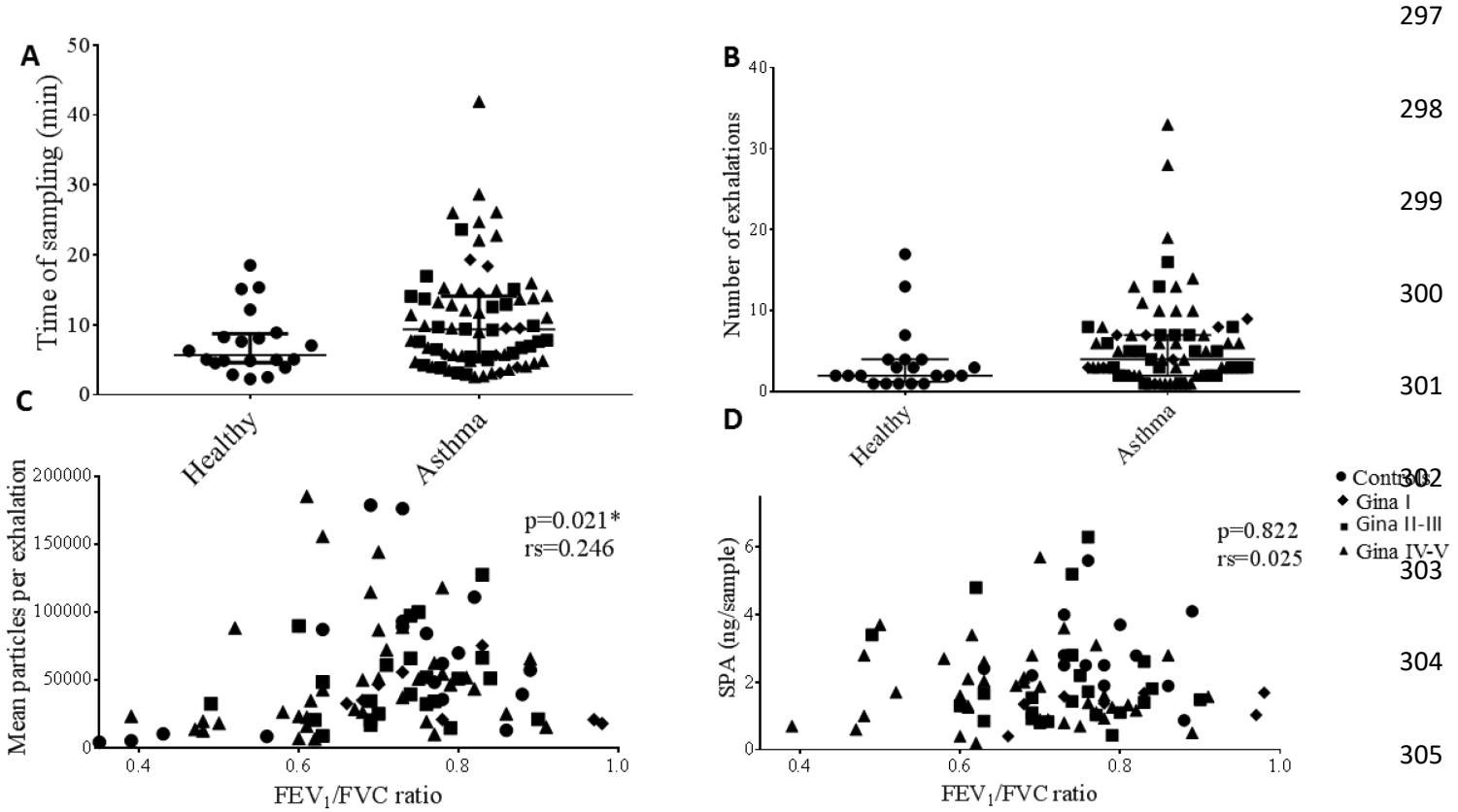


Figure 1 a, b, c, d: PExA sampling outcomes. (a) Number of exhalations in the controls group [5 (3-9)] and asthmatics [8 (5-12)]. (b) Time required to sample >30ng PEx in the controls group [6 (5-9)] and asthmatics [9 (5-14)]; (c) Relationship between mean number of particles per exhalation and FEV_1/FVC.(d) Relationship between total number of ng of SP-A per sample and FEV_1/FVC. Dots represent healthy controls, diamonds asthma GINA step treatment I, squares asthma GINA step treatment II and III and triangles asthma GINA step treatment IV and V.

Clinical Demographics of the Asthma Discovery Cohort

Basic demographic and clinical characteristics are summarised in **Table 1A**. GINA asthma severity groups were well matched for age, sex and asthma age of onset. In contrast, GINA IV-V asthmatics had significantly poorer spirometric lung function, a greater proportion of patients had a $FEV_1/FVC <$ lower limit of normal (LLN) and displayed multiple physiological features of small airways dysfunction (abnormal R5-R20, AX, Sacin and LCI, **Table 1B**) when compared to healthy volunteers

TABLE 1A: DEMOGRAPHIC AND CLINICAL DATA ACCORDING TO GINA STEP TREATMENT IN THE DISCOVERY POPULATION.

Clinical Characteristics	Healthy (n=20)	GINA I (n=9)	GINA II-III (n26)	GINA IV-V (n=40)	Kruskal-Wallis *P* value
Age (years)	53 (45-68)	45 (28-63)	61 (49-65)	62 (53-68)	0.139
Sex (male/female)¥	10/10	2/7	13/13	24/16	0.224
Age of asthma diagnosis		22 (13-48)	21 (6-49)	36 (18-49)	0.523
BMI (kg/m^2)	27 (25-30)	25 (22-28)	26 (23-32)	30 (27-33)	0.049
Pack year history	15 (2-32)	0	6 (1-7)	8 (2-12)	0.403
ACQ -6	-	0.3 (0.0-1.3)[e]	0.8 (0.8-1.9)	1.6 (0.8-2.2)	0.013
AQLQ	-	6.5 (5.7-6.9)[e]	6.2 (4.4-6.4)	5.3 (4.5-6.2)	0.036
Post BD FVC (L)	4.2 (3.2-4.7)	3.6 (3.0-4.4)	3.5 (2.9-4.5)	3.8 (3.0-4.3)	0.362
Post BD FEV_1 (L)	3.4 (2.5-3.6)	3.0 (2.1-3.4)	2.5 (2.1-3.0)	2.4 (2.1-2.9)[c]	0.007
FEV_1/FVC	0.77 (0.73-0.81)	0.73 (0.67-0.90)	0.74 (0.64-0.78)	0.68 (0.61-0.77)	0.015
FEV_1/FVC post BD LLN (≥/<)	15/1	6/3	17/6	21/17	0.043

Definition of abbreviations: GINA: Global Initiative for Asthma; BMI: body mass index; ACQ-6: six-point asthma control questionnaire; AQLQ: asthma quality of life questionnaire; FEV_1: forced expiratory volume in one second; FVC: forced vital capacity; BD: Bronchodilator.
Data expressed as median, Q1-Q3; **¥:** p value based on chi-square test; Kruskal Wallis test followed by Dunn's multiple comparisons tests significant difference ($p<0.05$) between: c- healthy and GINA IV-V; e- GINA I and GINA IV-V.

TABLE 1B: SMALL AIRWAYS PHYSIOLOGY AND BIOMARKERS DATA ACCORDING TO GINA STEP TREATMENT IN THE DISCOVERY POPULATION.

Physiology	Healthy (n=20)	GINA I (n=9)	GINA II-III (n26)	GINA IV-V (n=40)	Kruskal-Wallis *P* value
% PEx SPA	2.6 (2.1-4.0)	2.7 (1.8-2.8)	2.6 (1.9-3.2)	2.6 (1.6-3.2)	0.483
% PEx albumin	5.1 (5.1-6.8)	6.1 (4.9-7.6)	6.7 (3.9-8.1)	4.8 (3.7-6.7)	0.388
R5 (KPa.s.L^{-1})	0.30 (0.26-0.36)[c]	0.32 (0.25-0.38)	0.37 (0.29-0.52)	0.41 (0.34-0.51)	0.032
R20 (KPa.s.L^{-1})	0.28 (0.22-0.32)	0.30 (0.26-0.34)	0.31 (0.24-0.35)	0.32 (0.28-0.37)	0.190
R5-R20 (KPa.s.L^{-1})	0.03 (0.03-0.06)	0.02 (0.01-0.05)	0.07 (0.01-0.15)	0.08 (0.02-0.16)	0.044
R5-R20 % predicted	30 (0-82)	23 (14-54)	92 (24-151)	75 (17-154)	0.036
AX (Hz kPa.L^{-1})	0.20 (0.13-0.40)[c]	0.37 (0.15-0.56)	0.44 (0.20-2.00)	0.76 (0.20-1.69)	0.040
LCI	7.16 (6.65-7.95)[c]	7.02 (6.06-7.63)	7.81 (7.20-8.80)	8.28 (7.14-9.15)	0.008
Scond	0.026 (0.008-0.048)	0.023 (0.011-0.072)	0.039 (0.022-0.077)	0.03 (0.018-0.047)	0.200
Sacin	0.194 (0.115-0.322)[c]	0.345 (0.142-0.525)	0.304 (0.176-0.524)	0.316 (0.241-0.487)	0.033

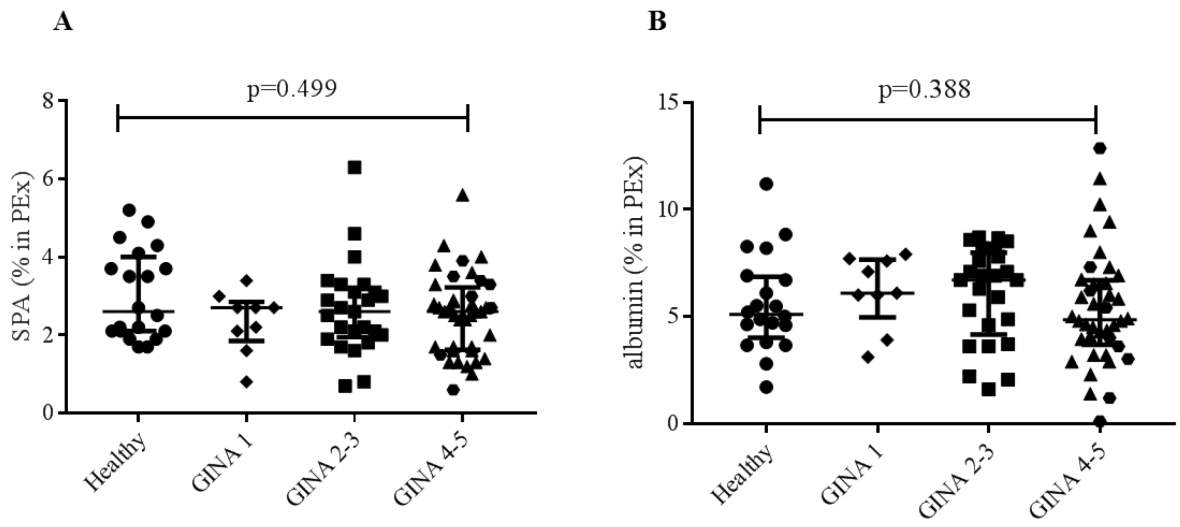
Definition of abbreviations: GINA: Global Initiative for Asthma; R5: resistance at 5Hz; R20: resistance at 20Hz; AX: Area of reactance; LCI: Lung clearance index; Scond: conductive ventilation heterogeneity; Sacin: acinar ventilation heterogeneity.
Data expressed as median, Q1-Q3; Kruskal Wallis test followed by Dunn's multiple comparisons tests, significant difference ($p<0.05$) between: c- healthy and GINA IV-V; e- GINA I and GINA IV-V.

% SPA and Albumin and asthma treatment intensity

SPA% and albumin % were not associated with GINA treatment intensity (**Table 1B, Figure 2a, b**) and did not differ between asthmatics and healthy volunteers. Neither protein biomarker correlated with standardised measures of asthma control or quality of life (ACQ-6 or AQLQ).

%SPA, Albumin and Small Airways Dysfunction Phenotypes

We evaluated the correlations between % SPA and % albumin and different demographics and small airway physiological measurements in the overall population (**Table 2**). Modest but significant correlations were found for %SPA with oscillometry parameters of small airways dysfunction (R5-R20 and AX): R5-R20 [both absolute value ($r=-0.256$, $p<0.05$) and % predicted ($r=-0.257$), $p<0.05$] and AX (-0.313, $p<0.05$). The spirometry measure of gas trapping – forced vital capacity -FVC (L) also demonstrated modest but significant correlations with % SPA ($r=0.287$; $p<0.05$). In contrast, % albumin demonstrated a significant correlation with FVC and GINA treatment intensity ($r=-0.285$, $p<0.05$) only. No correlations were observed between PExA protein concentrations and with multiple breath washout derived markers of small airways dysfunction (Scond or Sacin).



379

380 **Figure 2 a, b: SPA and albumin across GINA step treatments**. % of SPA (a) and albumin
381 (b) and across GINA groups. Dots (black) represent healthy controls, diamonds asthma GINA
382 step treatment I, squares asthma GINA step treatment II and III, triangles asthma GINA step
383 treatment IV and grey hexagons for V. Kruskal-Wallis *P* value across groups is as shown.

384

385

386

387

388

389

390

391

TABLE 2: % SPA AND ALBUMIN AND PHYSIOLOGICAL PARAMETERS

Variable	% SPA	% albumin
Age	-0.127	0.110
Height	0.171	0.091
BMI	-0.033	-0.044
ACQ-6	0.111	0.022
AQLQ	0.024	0.090
GINA	-0.006	**-0.285***
Sputum eosinophils$^{\Delta}$	0.012	0.018
Sputum epithelial cells$^{\Delta}$	-0.161	-0.152
R20	-0.097	-0.099
R5-R20	**-0.256***	-0.124
R5-R20 % predicted	**-0.257***	-0.101
AX	**-0.313****	-0.160
LCI	-0.159	0.076
Scond	-0.067	-0.045
Sacin	0.006	0.090
FVC Z score post BD	0.163	**0.246***
FVC post BD	**0.287***	**0.251***
FEV_1 Z score post BD	0.075	0.138
FEV_1 post BD	**0.308****	0.207

Definition of abbreviations: GINA: Global Initiative for Asthma; BMI: body mass index; ACQ-6: six-point asthma control questionnaire; AQLQ: asthma quality of life questionnaire; R5: resistance at 5Hz; R20: resistance at 20Hz; AX: Area of reactance; LCI: Lung clearance index; Scond: conductive ventilation heterogeneity; Sacin: acinar ventilation heterogeneity; FVC: forced vital capacity; FEV_1: Forced expiratory volume in one second;
Data expressed as Spearman r value. *p<0.05; ** p<0.01; ***p<0.001. Δ: data based on 40 patients (42% population).

Tables 3A, B and Figure 3 present summaries of the PExA protein and small airway physiology derived phenotypes using topological data analysis (TDA) applied to the overall discovery population This analysis excluded 4 patients which did not fit in any of the three TDA categories generated. Figure 3 demonstrates de TDA 2-dimentional networks of patients generated according to GINA step treatment (0-healthy volunteers), R5-R20 value, % SPA and % albumin. The round circles represent a network of patients with similar physiological outcomes. The lines connect network of patients (nodes) with similar outcomes.

As seen in tables 3 A and B, TDA group 3 had the highest proportion and majority (n=13/47) of healthy volunteer cases when compared to the TDA group 1 and 2. % SPA and % albumin levels were well preserved within group 3, which demonstrated normal oscillometry and MBW small airway indices as well as preserved spirometry, despite 17/47 patients being classified clinically as having severe asthma (GINA IV-V) based upon treatment requirements.

In contrast, TDA group 1 was comprised of very few healthy cases (n=2/29), with most patients at GINA treatment steps 3-5. Moreover, group 1 had significantly lower PExA % albumin and % SPA and concurrent evidence of abnormal post bronchodilator spirometry (FEV_1 and FVC) and small airway physiology (R5-R20, AX, Sacin) when compare to group 3 ($p<0.05$ for all comparisons). Group 1 also demonstrated the poorest asthma control and quality of life with statistically significant and clinically important (≥ 0.5 units) differences in standardised asthma control and quality of life marker (ACQ-6 and AQLQ) when compared to group 3.

Finally, group 2 represented an intermediate population with reference to groups 1 and 3, again primarily comprised of asthmatic subjects (n=13/15). Additionally, group 2 had the lowest numerical % SPA ($p<0.05$ vs group 1) but in contrast to group 1, a preserved % albumin, and had evidence of small airways dysfunction compared to group 1 (R5-R20, AX, and LCI, $p<0.05$) but demonstrated preserved post bronchodilator spirometry and comparable asthma control and quality of life (ACQ-6, AQLQ) to TDA group 1.

TABLE 3A – DEMOGRAPHIC AND CLINICAL DATA ACCORDING TO TDA GROUPS IN THE DISCOVERY POPULATION.

Clinical Characteristics	TDA group 1 **(n=29)**	TDA group 2 **(n=15)**	TDA group 3 **(n=47)**	*P* value
N per group (controls, GINA I, GINA II-III, GINA IV, GINA V)¥	29 (2, 2, 10, 11, 4)	15 (2, 1, 5, 7, 0)	47 (13, 6, 11, 12, 5)	0.287
% Healthy controls¥	6.9	13.3	27.6	0.066
Age (years)	62 (51-68)	65 (56-70)	59 (45-65)	0.0783
Sex (percent male)¥	55.17%	42.86%	51.06	0.7508
BMI (kg/m^2)	30 (26-34)	31 (23-34)	28 (24-30)	0.0911
Pack year smoking history	7.4 (5.0-11.0)	18.5 (3.0-34.0)	6.0 (1.0-9.0)	0.5991
GINA treatment step#	4 (3-4)	4 (3-4)	3.5 (2-4)	0.645
ACQ-6#	1.63 (0.67-2.16)	0.67 (0.28-2.67)	1.00 (0.66-1.67)	0.2727
AQLQ#	4.91 (4.12-6.03)	6.25 (5.70-6.69)	6.11 (5.21-6.47)	0.0059 1 vs. 2*,1 vs. 3*
Post BD FVC (L)	2.97 (2.49-3.57)	3.40 (2.77-3.99)	3.83 (3.46-4.45)	0.0007 1 vs. 3*
Post BD FEV$_1$ (L)	2.19 (1.91-2.44)	2.40 (1.89-3.05)	3.08 (2.65-3.90)	<.0001 1 vs. 3*
Post BD FEV1 Z score	-1.44 (-2.59- -0.03)	-0.64 (-1.29-0.49)	-0.49 (-1.25-0.69)	<0.0001 1 vs. 3*
Post BD FVC Z score	-0.64 (-1.46- -0.03)	-0.27 (-0.83-0.39)	0.06 (-0.25-0.83)	0.002 1 vs. 3*
FEV$_1$/FVC	0.69 (0.60-0.77)	0.72 (0.69-0.77)	0.73 (0.67-0.78)	0.2056
FEV$_1$/FVC post BD LLN (% above)	57%	78%	70%	0.3733

Definition of abbreviations: GINA: Global Initiative for Asthma; BMI: body mass index; ACQ-6: 6-point asthma control questionnaire; AQLQ: asthma quality of life questionnaire; FEV$_1$: forced expiratory volume in one second; FVC: forced vital capacity; BD: Bronchodilator. Data expressed as

median, Q1-Q3. All tests are Kruskal-Wallis unless stated otherwise. ¥ = Fisher's exact test (controls vs asthma); # only asthma.

TABLE 3B – SMALL AIRWAYS PHYSIOLOGY AND BIOMARKERS DATA ACCORDING TO TDA GROUPS IN THE DISCOVERY POPULATION

	TDA group 1 (n=29)	TDA group 2 (n=15)	TDA group 3 (n=47)	Kruskal-Wallis *P* value
% SPA	2.1 (1.4-2.7)	1.7 (1.6-2.4)	3.1 (2.6-3.8)	<.0001 1 vs. 3*,2 vs. 3*
% Albumin	3.9 (2.6-4.8)	5.6 (5.0-8.0)	6.7 (4.9-7.9)	<.0001 1 vs. 2*,1 vs. 3*
R5 (KPa.s.L^{-1})	0.51 (0.44-0.64)	0.39 (0.29-0.51)	0.32 (0.25-0.39)	<.0001 1* vs. 3*
R20 (KPa.s.L^{-1})	0.34 (0.32-0.38)	0.31 (0.28-0.37)	0.30 (0.23-0.35)	0.0654
R5-R20 (KPa.s.L^{-1})	0.15 (0.12-0.20)	0.07 (0.02-0.17)	0.02 (0.01-0.05)	<.0001 All Groups*
AX (Hz kPa.L^{-1})	1.69 (1.11-3.04)	0.64 (0.26-2.12)	0.20 (0.12-0.42)	<.0001 All Groups*
LCI	8.62 (7.54-9.63)	8.77 (7.26-9.07)	7.30 (7.01-7.99)	0.0019 1 vs. 3*,**2 vs. 3***
Scond	0.036 (0.018-0.069)	0.044 (0.018-0.073)	0.029 (0.014-0.044)	0.2688
Sacin	0.412 (0.231-0.529)	0.302 (0.094-0.486)	0.269 (0.169-0.355)	0.0184 1 vs. 3*

Definition of abbreviations: GINA: Global Initiative for Asthma; R5: resistance at 5Hz; R20: resistance at 20Hz; AX: Area of reactance; LCI: Lung clearance index; Scond: conductive ventilation heterogeneity; Sacin: acinar ventilation heterogeneity. Data expressed as median, Q1-Q3. All tests are Kruskal-Wallis unless stated otherwise. ¥ = Chi-Squared test.

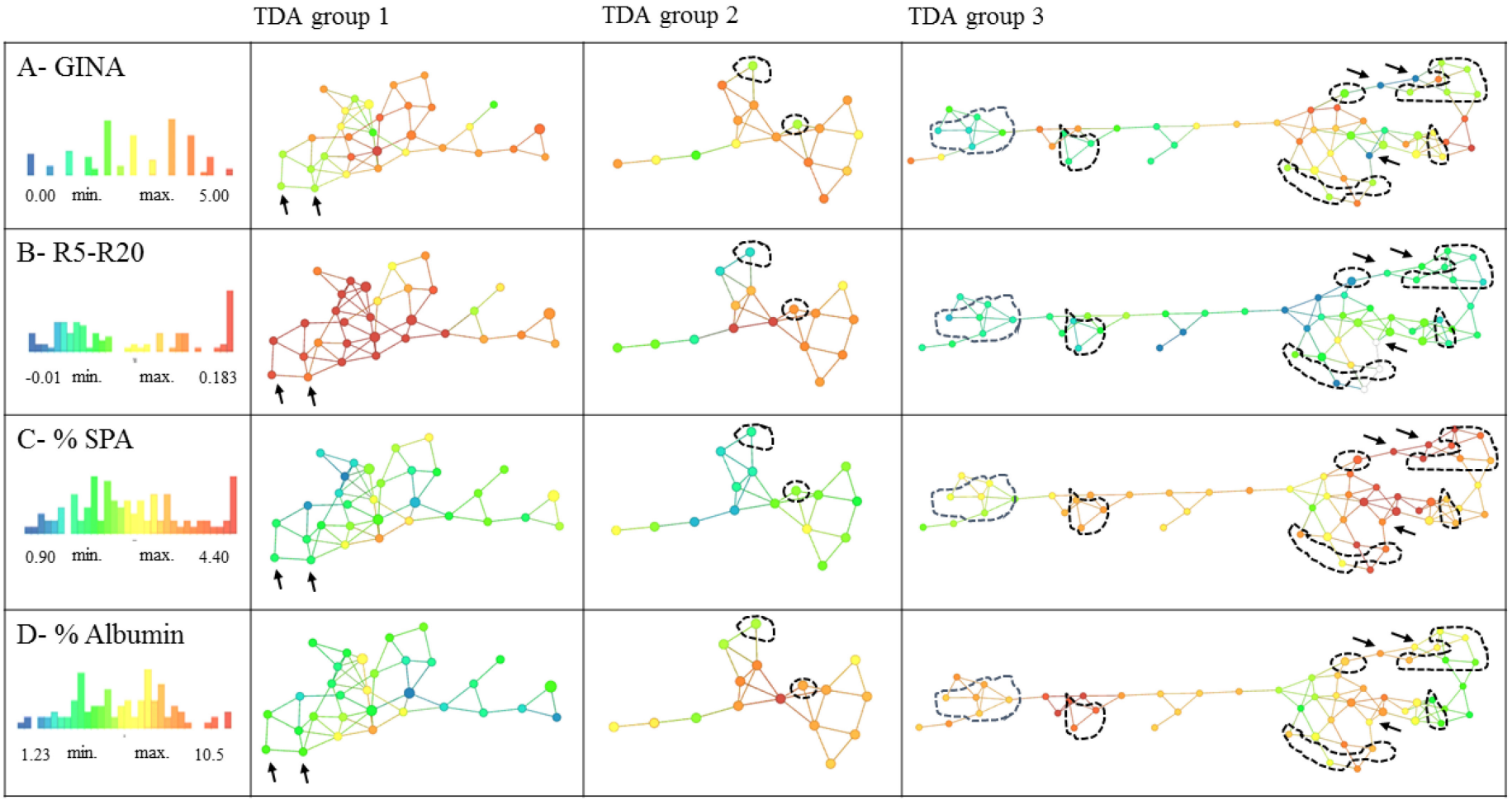

TDA group 1
TDA group 2
TDA group 3
A- GINA
0.00 min. max. 5.00
B- R5-R20
-0.01 min. max. 0.183
C- % SPA
0.90 min. max. 4.40
D- % Albumin
1.23 min. max. 10.5

Figure 3: TDA analysis. Image derived from TDA analysis, showing that three different groups were generated. Healthy controls and asthma patients were clustered and annotated by the following parameters: A) GINA treatment step treatment (red- only asthma patients, 0-healthy controls only); B) R5-R20 (red-very high frequency dependence of resistance) C) %SPA (red- higher levels of SPA) and D) %albumin (red-higher levels of albumin). There was a further group of four patients not fitting in any category and therefore not included in the analysis. TDA group 1 is mainly small airways disease predominant, with low % of SPA and albumin (2 healthy controls, 27 asthmatics); TDA group 2 is an intermediate group, with low % SPA but preserved albumin (2 healthy controls, 13 asthmatics); TDA group 3 demonstrate overall normal small airway lung function and higher SPA and albumin % (13 healthy controls, 34 asthmatics). Arrows and bounded shapes represent nodes with only healthy controls and nodes with high number of healthy controls, respectively**.** It can be seen that the majority of heathy patients are located within group 3, with occasional and scarce cases within group 1 and 2.

Replication Cohort

In the replication and discovery asthma populations (**Figure 4 a and c**), % SPA correlated with absolute FVC (rs= 0.378, p=0.001 discovery and rs=0.543; p=0.001 replication).

For % albumin (**figure 4 b and d**), only the discovery cohort demonstrated correlations with absolute FVC (rs=0.261, p=0.032). The replication cohort demonstrated a non-statistical, but visual trend for association between FVC and % albumin (**Figure 4 b and** d).

In contrast to the discovery cohort, we did not identify significant correlations between R5-R20 and either % SPA or % albumin in PEx, however, the trend was similar, higher values of R5-R20 associated with lower % SPA.

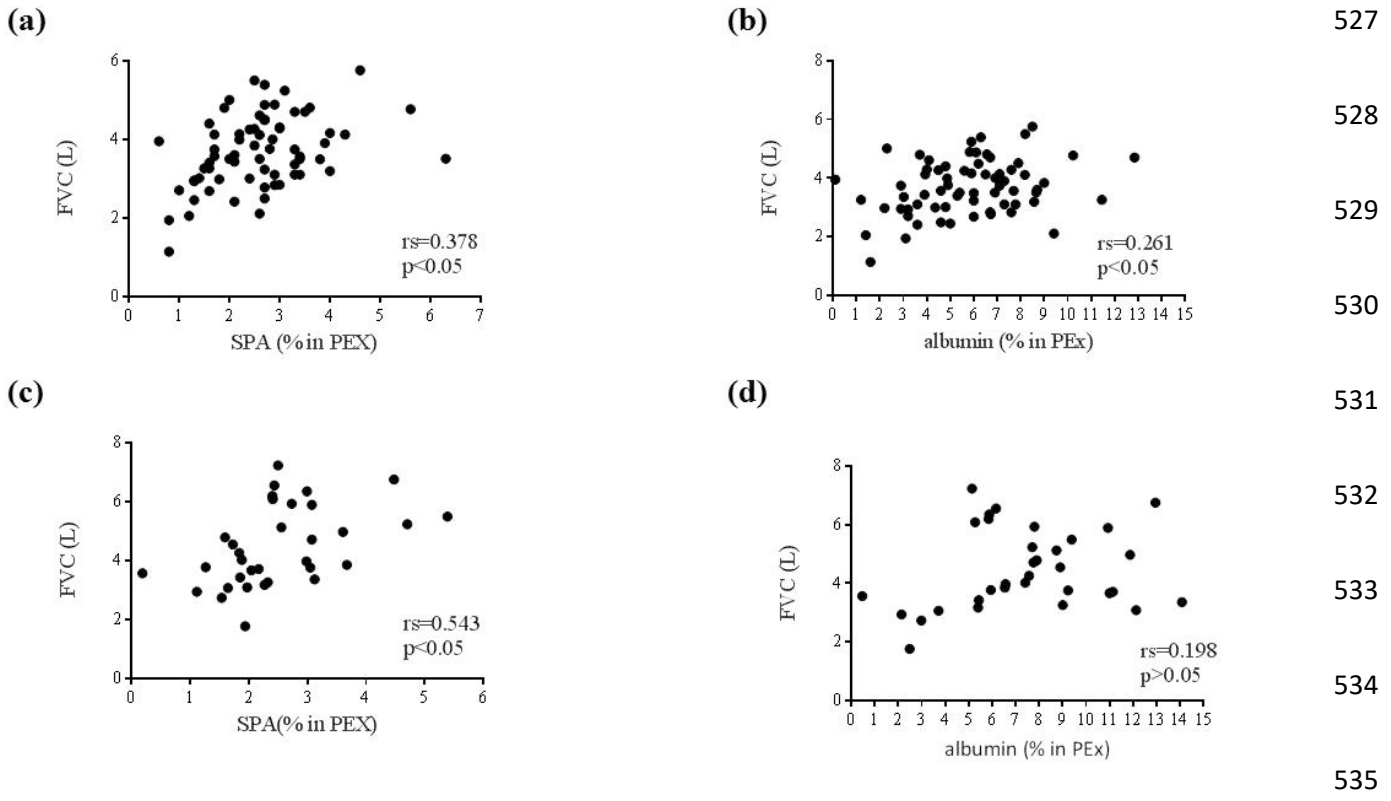


Figure 4 a, b, c, d: PEx and FVC in the replication cohort. Correlation between absolute FVC (Forced Vital Capacity) and %SPA and % of albumin, in the discovery population (4a, 4b) and replication population (4c, 4d).

Discussion

We have shown for the first time that PExA sampling method is feasible across different asthma severity and that sufficient quantities of PEx can be sampled to allow protein biomarker analysis.

Furthermore, we have demonstrated using unbiased statistical phenotyping with TDA analysis that there appear to be phenotypes of patients with low % SPA and/or % albumin values, comprising primarily asthmatic subjects with concurrently abnormal small airway physiological indices captured by IOS and MBW. One of the small airway disease phenotypes had clinically important disease (assessed by validated patient reported outcome measures) when compared to the phenotype of patients without small airway abnormalities. A further phenotype appeared to have well-controlled asthma and spirometry with isolated physiological abnormalities in the small airways. Lower % SPA values were a reconciling feature of both small airway phenotypes, suggesting that the deficiency in SPA may be possibly be causal to airways closure in the small airways.

Finally, we have identified that the % SPA is associated with FVC, a marker of airways closure and gas trapping [38] in both a discovery and an independent replication population.

The ability to measure protein biomarkers from the smaller airways offers a window of opportunity to study the pathobiology of small airways disease. Furthermore, low SPA levels in PEx, corrected for acquired particle mass and potential bias due to sampling in the context of airflow obstruction, was not only associated with small airways dysfunction/airway closure captured by IOS, MBW and FVC measurements, but also identified two phenotypes of patients with multiple physiological markers of small airways dysfunction and impaired asthma control/quality of life. These findings not only add credence to the notion that PEx samples originate from the smaller airways but also for a potential causal association between

SPA deficiency and small airways dysfunction/ airways closure. The small airway phenotype extended across the spectrum of asthma severity and was not associated with eosinophilic airways inflammation (See online supplement), suggesting that it may require alternative approaches of treatment that extend beyond inhaled corticosteroids and drugs that modify type 2 inflammation in asthma.

There is compelling evidence from animal models and observational studies, linking SPA to asthmatic airway dysfunction and inflammation. Genetic variation of SPA has been shown to alter host immunological response to bacterial infection with mycoplasma in asthma [39], which may in turn be regulated by mast cells TNF receptor expression. These observations are further supported by animal models of SPA knockout that suggest that SPA deficiency amplifies allergic CD4 T cell driven airway inflammation [40]. Ledford *et al* have recently reported in a systematic review that SPA/D are dysregulated in eosinophil-dominated inflammatory diseases, suggesting a therapeutic potential role of SPA/D, yet to be tested in humans [41]. Broncho alveolar lavage studies in asthma and healthy report conflicting data on the role and concentration of SPA [42-43], highlighting the potential limitations of BAL in measuring protein biomarkers in asthma.

Our findings and previous literature on PExA, would suggest that PEx %SPA in asthma reflect protein concentrations in the small airways lining fluid and that deficiency of this protein may then directly promote airway closure and ventilation heterogeneity. There is some evidence of beneficial outcomes derived from inhaled synthetic surfactant in allergic asthma [44], and PEx SPA quantification may provide an opportunity to stratify patients for SPA targeted intervention trials in the future.

We utilised a combination of IOS, MBW and FVC to measure small airways dysfunction and airways closure, respectively. It is well recognised that small airways dysfunction associates

with both asthma symptoms and key asthma traits e.g. hyper responsiveness [45]. A recent systematic review has highlighted the evidence supporting R5-R20 as a small airway detection tool in asthma [46]. Reduction in FVC has been associated with bronchoconstriction occurring due to airway closure and increased levels of airway hyperresponsiveness [38, 47]. Therefore, our findings that FVC was proportional to the % of SPA and albumin found in PEx, provides evidence that small airway closure may be due to an alteration in protein composition in the airway surface liquid in asthma.

Previous studies have demonstrated that treatment with both inhaled and oral steroids in asthma attenuates plasma protein and albumin in sputum supernatants in asthma [48]. In support of this, TDA group 3 demonstrated lower numerical median GINA treatment and preserved % albumin compared to TDA group 1 who had both a low % albumin and % SPA. However, both hypothesis that albumin in the RTLF could be increased or decreased due to airway inflammation should be considered. Higher levels of albumin levels may equate to increased vascular permeability or transudation of albumin due to increased hydrostatic pressure, but the exact mechanist requires further research.

Limitations of our study include the relatively small sample size – nonetheless this study represents the largest study to apply the PExA technique to a well characterised cohort of adult asthmatics to date. Moreover, further studies are required to identify the relationship between protein marker of type 2 inflammation in PExA and SPA% in asthma. Studies that directly compare SPA concentration in sputum supernatant and PEx would further add to the validation of PEx as a small airway specific matrix. Of note however, in a separate adult asthma population of 42 individuals across different severities [GINA I-V], %SPA and absolute SPA in PEx did not correlate with serum SPA ($p=0.265$, $p=0.579$ respectively, data not shown).

Furthermore additional validation of SPA and albumin in PEx are required with respect to measurement repeatability and healthy population normative ranges.

We conclude that the PExA method has the potential to non-invasively sample the small airways derived proteins in asthma (including severe asthmatics with high unmet need) and that two exemplar PExA proteins (SPA and albumin) are associated with small airway dysfunction phenotypes in asthma. Further studies are now required to reproduce our findings and further develop PEx as a novel matrix to study small airway biology non-invasively.

Sources of support/ Acknowledgments: Supported by an unrestricted grant from the Chiesi Onulus Foundation (Validation of Particle in Exhaled Air (PEx) as a Novel Matrix for Non-

Invasive Detection of Small Airways Disease in Asthma) and additional support from the Leicester National Institute for Health Research (NIHR) Biomedical Research Centre: Respiratory Theme. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

The authors would like to thank Ayasdi (Menlo Park, California) and Dr Devi Ramanan for access to the Ayasdi CORE and Python SDK platforms for data analysis.

This article contains online supplementary material.

References

[1] Global Strategy for Asthma Management and Prevention. Global Initiative for Asthma (GINA) 2012. Available from: http://www.ginasthma.org/.

[2] Benayoun L, Druilhe A, Dombret MC, Aubier M, Pretolani M. Airway structural alterations selectively associated with severe asthma. Am J Respir Crit Care Med. 2003 May 15;167(10):1360-8.

[3] Lipworth B, Manoharan A, Anderson W. Unlocking the quiet zone: the small airway asthma phenotype. Lancet Respir Med. 2014 Jun 30;2(6):497-506.

[4] Wahidi MM, Rocha AT, Hollingsworth JW, Govert JA, Feller-Kopman D, Ernst A. Contraindications and safety of transbronchial lung biopsy via flexible bronchoscopy. Respiration. 2005 Jun 2;72(3):285-95.

[5] Reynolds HY. Bronchoalveolar lavage and other methods to define the human respiratory tract milieu in health and disease. Lung. 2011 Apr 1;189(2):87-99.

[6] Almstrand AC, Ljungström E, Lausmaa J, Bake B, Sjövall P, Olin AC. Airway monitoring by collection and mass spectrometric analysis of exhaled particles. Anal Chem. 2008 Dec 5;81(2):662-8.

673 [7] Bredberg A, Gobom J, Almstrand AC, Larsson P, Blennow K, Olin AC, Mirgorodskaya
674 E. Exhaled endogenous particles contain lung proteins. Clin Chem. 2012 Feb 1;58(2):431-40.
675
676 [8] Larsson P, Lärstad M, Bake B, Hammar O, Bredberg A, Almstrand AC, Mirgorodskaya E,
677 Olin AC. Exhaled particles as markers of small airway inflammation in subjects with asthma.
678 Clin Physiol Funct Imaging. 2015 Nov 1.
679
680 [9] Larsson P, Mirgorodskaya E, Samuelsson L, Bake B, Almstrand AC, Bredberg A, Olin
681 AC. Surfactant protein A and albumin in particles in exhaled air. Resp Med. 2012 Feb
682 29;106(2):197-204.
683
684 [10] Lärstad M, Almstrand AC, Larsson P, Bake B, Larsson S, Ljungström E, Mirgorodskaya
685 E, Olin AC. Surfactant Protein A in Exhaled Endogenous Particles Is Decreased in Chronic
686 Obstructive Pulmonary Disease (COPD) Patients: A Pilot Study. PloS One. 2015;10(12).
687
688 [11] Almstrand AC, Josefson M, Bredberg A, Lausmaa J, Sjövall P, Larsson P, Olin AC.
689 TOF-SIMS analysis of exhaled particles from patients with asthma and healthy controls. Eur
690 Respir J. 2012 Jan 1;39(1):59-66.
691
692 [12] Bredberg A, Josefson M, Almstrand AC, Lausmaa J, Sjövall P, Levinsson A, Larsson P,
693 Olin AC. Comparison of exhaled endogenous particles from smokers and non-smokers using
694 multivariate analysis. Respiration. 2013 Jun 28;86(2):135-42.

[13] Holmgern H, Ljungström E, Almstrand AC, Bake B, Olin AC. Size distribution of exhaled particles in the range from 0.01 to 2.0 mm. J Aerosol Sci. 2010;41:439-46.

[14] Holmgren H, Gerth E, Ljungström E, Larsson P, Almstrand AC, Bake B, Olin AC. Effects of breath holding at low and high lung volumes on amount of exhaled particles. Respir Physiol Neurobiol. 2013 Jan 15;185(2):228-34.

[15] Larsson P, Bake B, Wallin A, Hammar O, Almstrand AC, Lärstad M, Ljungström E, Mirgorodskaya E, Olin AC. The effect of exhalation flow on endogenous particle emission and phospholipid composition. Respiratory physiology & neurobiology. 2017 Sep 1;243:39-46.

[16] Ljungkvist G, Ullah S, Tinglev Å, Stein K, Bake B, Larsson P, Almstrand AC, Viklund E, Hammar O, Sandqvist S, Beck O. Two techniques to sample non-volatiles in breath—exemplified by methadone. J Breath Res. 2017 Dec 8;12(1):016011.

[17] Wang Y, Voelker DR, Lugogo NL, Wang G, Floros J, Ingram JL, Chu HW, Church TD, Kandasamy P, Fertel D, Wright JR. Surfactant protein A is defective in abrogating inflammation in asthma. Am J of Physiol Lung Cell Mol Physiol. 2011 Oct 1;301(4): L598-606.

[18] Wang JY, Kishore U, Lim BL, Strong P, Reid KB. Interaction of human lung surfactant proteins A and D with mite (Dermatophagoides pteronyssinus) allergens. Clinical & Experimental Immunology. 1996 Nov 1;106(2):367-73.

[19] Hohlfeld JM, Erpenbeck VJ, Krug N. Surfactant proteins SP-A and SP-D as modulators of the allergic inflammation in asthma. Pathobiology. 2002;70(5):287-92.

[20] Anzueto A, Jubran A, Ohar JA, Piquette CA, Rennard SI, Colice G, Pattishall EN, Barrett J, Engle M, Perret KA, Rubin BK. Effects of aerosolized surfactant in patients with stable chronic bronchitis: a prospective randomized controlled trial. JAMA. 1997 Nov 5; 278 (17): 1426-31.

[21] Khor YH, Teoh AK, Lam SM, Mo DC, Weston S, Reid DW, Walters EH. Increased vascular permeability precedes cellular inflammation as asthma control deteriorates. Clin Exp Allergy. 2009 Nov 1;39(11):1659-67.

[22] Ericson PA, Mirgorodskaya E, Hammar OS, Viklund EA, Almstrand AC, Larsson PJ, Riise GC, Olin AC. Low Levels of Exhaled Surfactant Protein A Associated With BOS After Lung Transplantation. Transplantation direct. 2016 Sep;2(9).

738 [23] Kjellberg S, Houltz BK, Zetterström O, Robinson PD, Gustafsson PM. Clinical
739 characteristics of adult asthma associated with small airway dysfunction. Respir Med. 2016
740 Aug 31;117:92-102.

741

742 [24] Juniper EF, Svensson K, Mörk AC, Ståhl E. Measurement properties and interpretation
743 of three shortened versions of the asthma control questionnaire. Respir Med. 2005; 99(5):
744 553-8.

745

746 [25] Juniper EF, Guyatt GH, Ferrie PJ, King DR. Development and validation of a
747 questionnaire to measure asthma control. Eur Respir J. 1999 Oct 1;14(4):902-7.

748

749 [26] Brightling CE, Ward R, Woltmann G, Bradding P, Sheller JR, Dworski R, Pavord ID.
750 Induced sputum inflammatory mediator concentrations in eosinophilic bronchitis and asthma
751 Am J Respir Crit Care Med. 2000 Sep 1;162(3):878-82.

752

753 [27] Miller MR, Hankinson JA, Brusasco V, Burgos F, Casaburi R, Coates A, Crapo R,
754 Enright P, Van Der Grinten CP, Gustafsson P, Jensen R. Standardisation of spirometry. Eur
755 Respir J. 2005 Aug 1;26(2):319-38.

756

757 [28] Oostveen E, MacLeod D, Lorino H, Farre R, Hantos Z, Desager K, Marchal F. The
758 forced oscillation technique in clinical practice: methodology, recommendations and future
759 developments. Eur Respir J. 2003 Dec 1;22(6):1026-41.

[29] Gonem S, Natarajan S, Desai D, Corkill S, Singapuri A, Bradding P, Gustafsson P, Costanza R, Kajekar R, Parmar H, Brightling CE. Clinical significance of small airway obstruction markers in patients with asthma. Clin Exp Allergy. 2014 Apr 1;44(4):499-507.

[30] Gonem S, Singer F, Corkill S, Singapuri A, Siddiqui S, Gustafsson P. Validation of a photoacoustic gas analyser for the measurement of functional residual capacity using multiple-breath inert gas washout. Respiration. 2014 Feb 13;87(6):462-8.

[31] Gonem S, Hardy S, Buhl N, Hartley R, Soares M, Kay R, Costanza R, Gustafsson P, Brightling CE, Owers-Bradley J, Siddiqui S. Characterization of acinar airspace involvement in asthmatic patients by using inert gas washout and hyperpolarized 3 helium magnetic resonance. J Allergy Clin Immunol. 2016 Feb 29;137(2):417-25.

[32] BioVendor. Human Surfactant Protein A ELISA Product Data Sheet. Brno; 2009

[33] Immunology Consultants Laboratory Inc. Human Albumin Immunoperoxidase Assay for Determination of Albumin In Human Samples. Portland, USA, Version3.

[34] Siddiqui S, Shikotra A, Richardson M, Doran E, Choy D, Bell A, Austin C, Eastham-Anderson J, Hargadon B, Arron J, Wardlaw A, Brightling C, Heaney L, Bradding P. Airway

781 Pathological Heterogeneity in Asthma: Visualisation of Disease Micro-Clusters using
782 Topological Data Analysis. J Allergy Clin Immunol, 2018 Mar 14.

783

784 [35] Carlsson G. Topology and data. Bull Amer Math Soc. 2009;46(2):255-308.

785

786 [36] Lum PY, Singh G, Lehman A, Ishkanov T, Vejdemo-Johansson M, Alagappan M,
787 Carlsson J, Carlsson G. Extracting insights from the shape of complex data using topology.
788 Sci Rep. 2013 Feb 7;3:1236.

789

790 [37] Sarikonda G, Pettus J, Phatak S, Sachithanantham S, Miller JF, Wesley JD, Cadag E,
791 Chae J, Ganesan L, Mallios R, Edelman S. CD8 T-cell reactivity to islet antigens is unique to
792 type 1 while CD4 T-cell reactivity exists in both type 1 and type 2 diabetes. J Autoimm. 2014
793 May 1;50:77-82.

794

795

796 [38] Sorkness RL, Bleecker ER, Busse WW, Calhoun WJ, Castro M, Chung KF, Curran-
797 Everett D, Erzurum SC, Gaston BM, Israel E, Jarjour NN. Lung function in adults with stable
798 but severe asthma: air trapping and incomplete reversal of obstruction with bronchodilation.
799 Journal of applied physiology. 2008 Feb;104(2):394-403.

800

801

802 [39] Ledford JG, Voelker DR, Addison KJ, Wang Y, Nikam VS, Degan S, Kandasamy P,
803 Tanyaratsrisakul S, Fischer BM, Kraft M, Hollingsworth JW. Genetic variation in SP-A2

804 leads to differential binding to Mycoplasma pneumoniae membranes and regulation of host
805 responses. Journal Immunol. 2015 Jun 15;194(12):6123-32.

806

807 [40] Scanlon ST, Milovanova T, Kierstein S, Cao Y, Atochina EN, Tomer Y, Russo SJ, Beers
808 MF, Haczku A. Surfactant protein-A inhibits Aspergillus fumigatus-induced allergic T-cell
809 responses. Respir Res. 2005 Aug 24;6(1):97

810

811 [41] Ledford JG, Addison KJ, Foster MW, Que LG. Eosinophil-associated lung diseases. A
812 cry for surfactant proteins A and D help?. Am J Respir Cell Mol Biol. 2014 Nov;51(5):604-
813 14.

814

815 [42] Cheng G, Ueda T, Numao T, Kuroki Y, Nakajima H, Fukushima Y, Motojima S, Fukuda
816 T. Increased levels of surfactant protein A and D in bronchoalveolar lavage fluids in patients
817 with bronchial asthma. Eur Respir J. 2000 Nov 1;16(5):831-5.

818

819 [43] Van De Graaf EA, Jansen HM, Lutter R, Alberts C, Kobesen J, de Vries IJ, Out TA.
820 Surfactant protein A in bronchoalveolar lavage fluid. J Lab Clin Med. 1992 Aug;120(2):252-
821 63.

822

823 [44] Babu KS, Woodcock DA, Smith SE, Staniforth JN, Holgate ST, Conway JH. Inhaled
824 synthetic surfactant abolishes the early allergen-induced response in asthma. Eur Respir J.
825 2003 Jun 1;21(6):1046-9.

826

827 [45] Hamid Q, Song Y, Kotsimbos TC, Minshall E, Bai TR, Hegele RG, Hogg JC.
828 Inflammation of small airways in asthma. J Allergy Clin Immunol. 1997 Jul 31;100(1):44-51.

829

830 [46] Galant SP, Komarow HD, Shin HW, Siddiqui S, Lipworth BJ. The case for impulse
831 oscillometry in the management of asthma in children and adults. Ann Allergy Asthma
832 Immunol. 2017 Jun 30;118(6):664-71.

833

834 [47] Chapman DG, Berend N, King GG, Salome CM. Increased airway closure is a
835 determinant of airway hyperresponsiveness. Eur Respir J. 2008 Dec 1;32(6):1563-9.

836

837 [48] Belda J, Margarit G, Martínez C, Bellido-Casado J, Casan P, Torrejón M, Brufal M,
838 Rodríguez-Jerez F, Sanchis J. Anti-inflammatory effects of high-dose inhaled fluticasone
839 versus oral prednisone in asthma exacerbations. Eur Respir J. 2007 Dec 1;30(6):1143-9.