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Particles in exhaled air (PExA): non-invasive phenotyping of small airways disease in adult asthma

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

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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.

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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.

65 Abbreviations:

66 ACQ: Asthma control Questionnaire

67 AQLQ: Asthma Quality of Life Questionnaire

68 AX: Area of Reactance

69 BD: Bronchodilator

70 BMI: Body Mass Index

71 BOS: Bronchiolitis obliterans syndrome

72 FEV₁: Forced Expiratory Volume in the first second

73 FVC: Forced Vital Capacity

74 GINA: Global Initiative for Asthma

75 IOS: Impulse Oscillometry

76 LCI: Lung Clearance Index

77 MBW: Multiple Breath Washout

78 OS: online supplement

79 PBS: Phosphate-Buffered Saline

80 PC₂₀: Methacholine Challenge test derived provocation concentration causing a 20% decrease
81 in FEV₁ from baseline

82 PExA: Particles in Exhaled Air method

83 PEx: Particles in Exhaled Air

84 Sacin: Acinar Ventilation Heterogeneity

85 Scond: Conductive Ventilation Heterogeneity

86 SPA: Surfactant protein A

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

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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.

118 Introduction

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

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

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

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

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

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

159 Previous studies have evaluated pooled PEx samples for SPA and albumin % in both mild
160 asthma and obstructive lung diseases (COPD and bronchiolitis obliterans syndrome-BOS)
161 [12, 14, 22]. These studies suggest that SPA is associated with obstructive lung disease COPD
162 and BOS, providing a sound justification to study the role of SPA and albumin in asthma and
163 their association with airways dysfunction.

164 In the present study, we hypothesised that: a) PExA method is both feasible across the
165 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 $\leq$ 8mg/ml, bronchodilator reversibility to 400 mcg of inhaled salbutamol of FEV₁ $\geq$ 12% and 200mls or peak flow variability of $\geq$ 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.

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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 County 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.

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PEx was extracted using PBS/0.05% Tween and particles SPA/Albumin evaluated using the Human Surfactant Protein A ELISA (BioVendor, Heidelberg, Germany), with monoclonal 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

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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 (FEV₁/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.

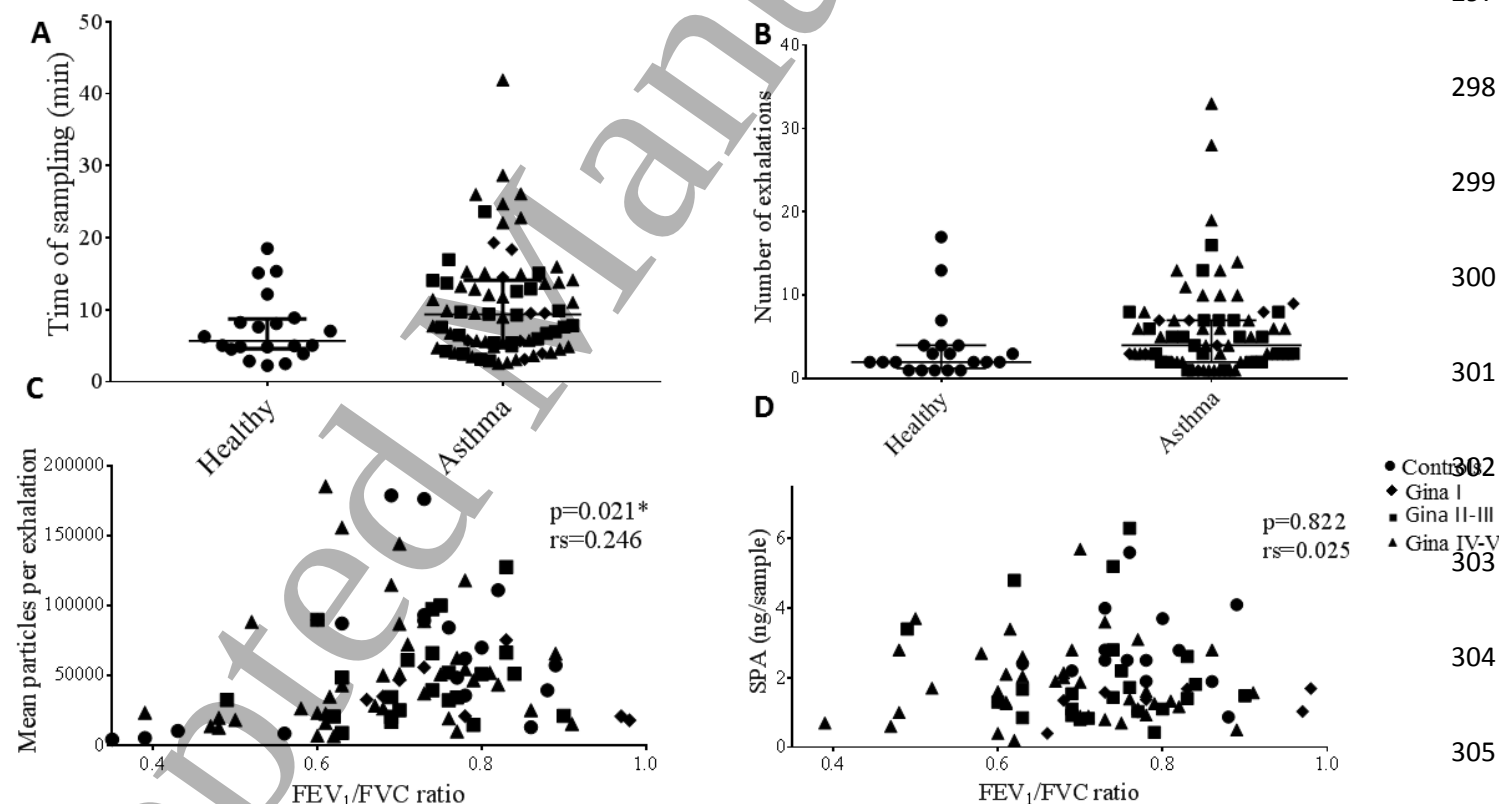


Figure 1 a, b, c, d: PExA sampling outcomes. (a) Number of exhalations in the controls group [5 (3-9)] and asthmatics [8 (5-12)]. (b) Time required to sample >30ng PEx in the controls group [6 (5-9)] and asthmatics [9 (5-14)]; (c) Relationship between mean number of particles per exhalation and FEV₁/FVC. (d) Relationship between total number of ng of SP-A per sample and FEV₁/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.

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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 FEV₁/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 (n=26)	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/m²)	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 FEV₁ (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
FEV₁/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
FEV₁/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; FEV₁: 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.

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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 (n=26)	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.

357 % SPA and Albumin and asthma treatment intensity

358 SPA% and albumin % were not associated with GINA treatment intensity (**Table 1B, Figure**
359 **2a, b**) and did not differ between asthmatics and healthy volunteers. Neither protein
360 biomarker correlated with standardised measures of asthma control or quality of life (ACQ-6
361 or AQLQ).

363 %SPA, Albumin and Small Airways Dysfunction Phenotypes

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

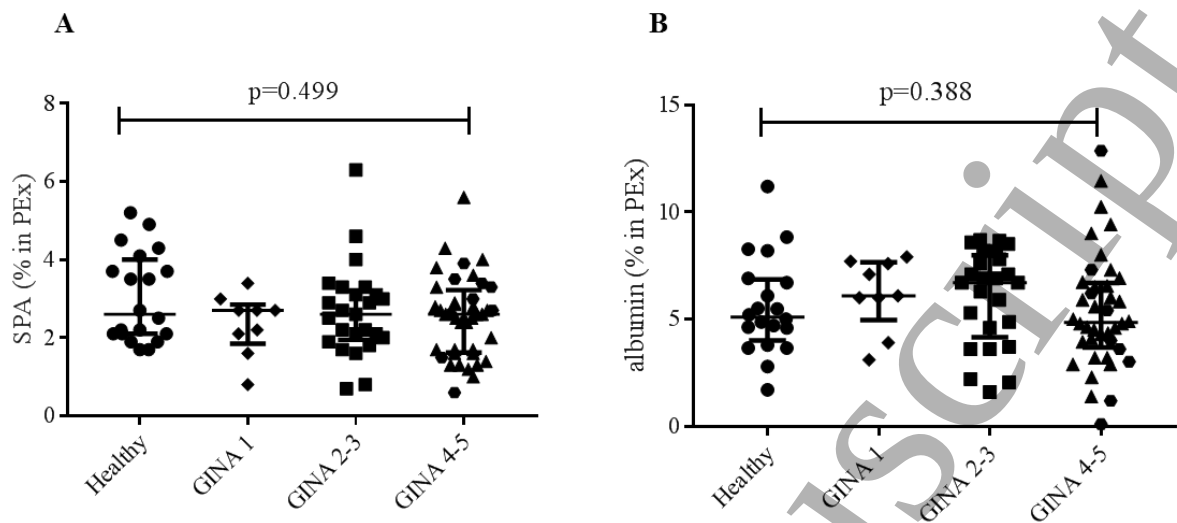


Figure 2 a, b: SPA and albumin across GINA step treatments. % of SPA (a) and albumin (b) and across GINA groups. Dots (black) represent healthy controls, diamonds asthma GINA step treatment I, squares asthma GINA step treatment II and III, triangles asthma GINA step treatment IV and grey hexagons for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

392 **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 ^Δ	0.012	0.018
Sputum epithelial cells ^Δ	-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

393 *Definition of abbreviations:* GINA: Global Initiative for Asthma; BMI: body mass index; ACQ-6: six-
394 point asthma control questionnaire; AQLQ: asthma quality of life questionnaire; R5: resistance at 5Hz;
395 R20: resistance at 20Hz; AX: Area of reactance; LCI: Lung clearance index; Scond: conductive
396 ventilation heterogeneity; Sacin: acinar ventilation heterogeneity; FVC: forced vital capacity; FEV₁:
397 Forced expiratory volume in one second;
398 Data expressed as Spearman r value. *p<0.05; ** p<0.01; ***p<0.001. Δ: data based on 40 patients
399 (42% population).

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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 (FEV₁ 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.

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3 426 Finally, group 2 represented an intermediate population with reference to groups 1 and 3,
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5 427 again primarily comprised of asthmatic subjects (n=13/15). Additionally, group 2 had the
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7 428 lowest numerical % SPA ($p<0.05$ vs group 1) but in contrast to group 1, a preserved %
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9 429 albumin, and had evidence of small airways dysfunction compared to group 1 (R5-R20, AX,
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11 430 and LCI, $p<0.05$) but demonstrated preserved post bronchodilator spirometry and comparable
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13 431 asthma control and quality of life (ACQ-6, AQLQ) to TDA group 1.
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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 FEV ₁ 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; FEV₁: forced expiratory volume in one second; FVC: forced vital capacity; BD: Bronchodilator. Data expressed as

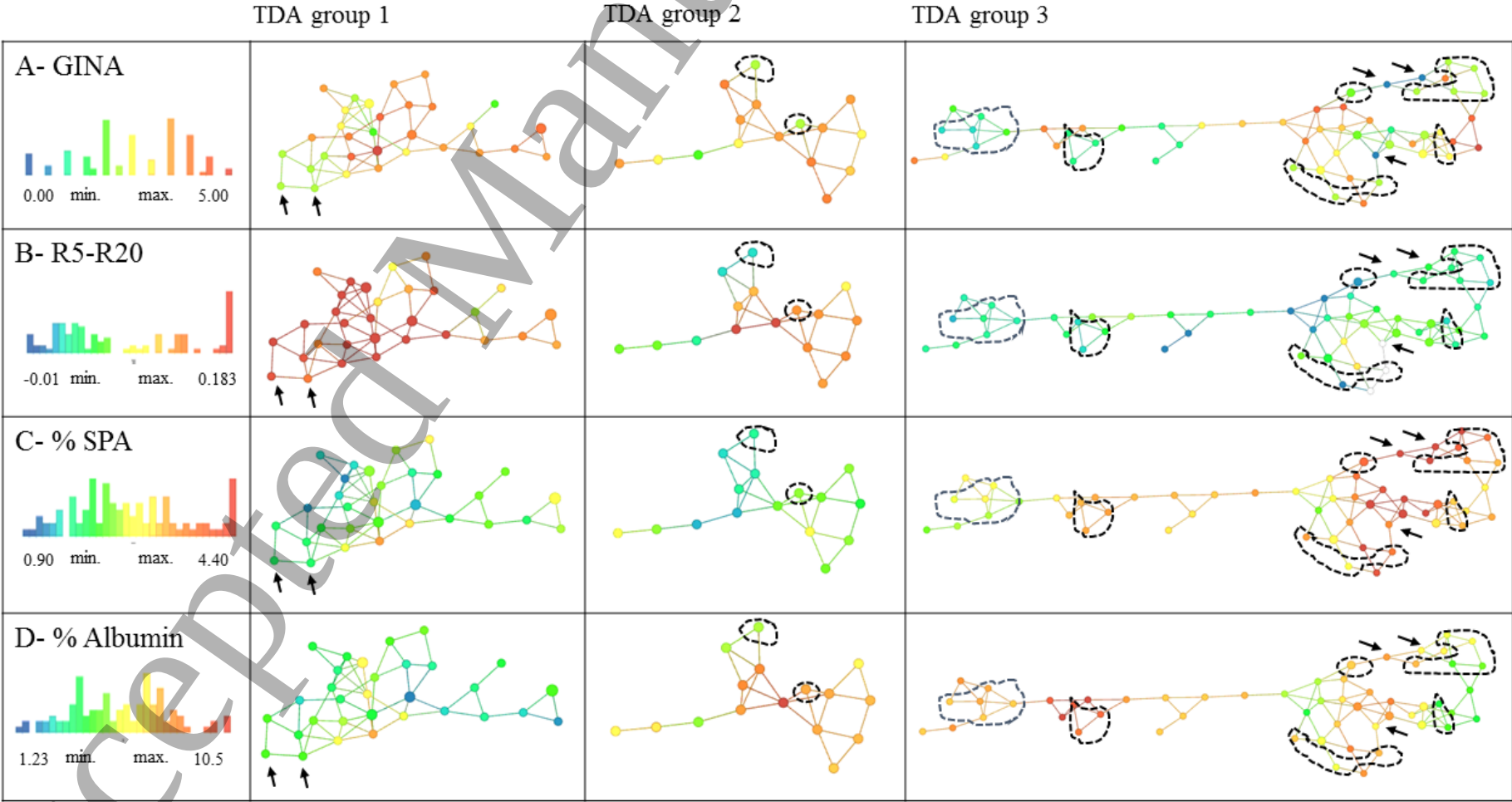
median, Q1-Q3. All tests are Kruskal-Wallis unless stated otherwise. ¥ = Fisher's exact test (controls vs asthma); # only asthma.

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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.



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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 healthy 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 ($r_s = 0.378$, $p = 0.001$ discovery and $r_s = 0.543$; $p = 0.001$ replication).

For % albumin (**figure 4 b and d**), only the discovery cohort demonstrated correlations with absolute FVC ($r_s = 0.261$, $p = 0.032$). 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.

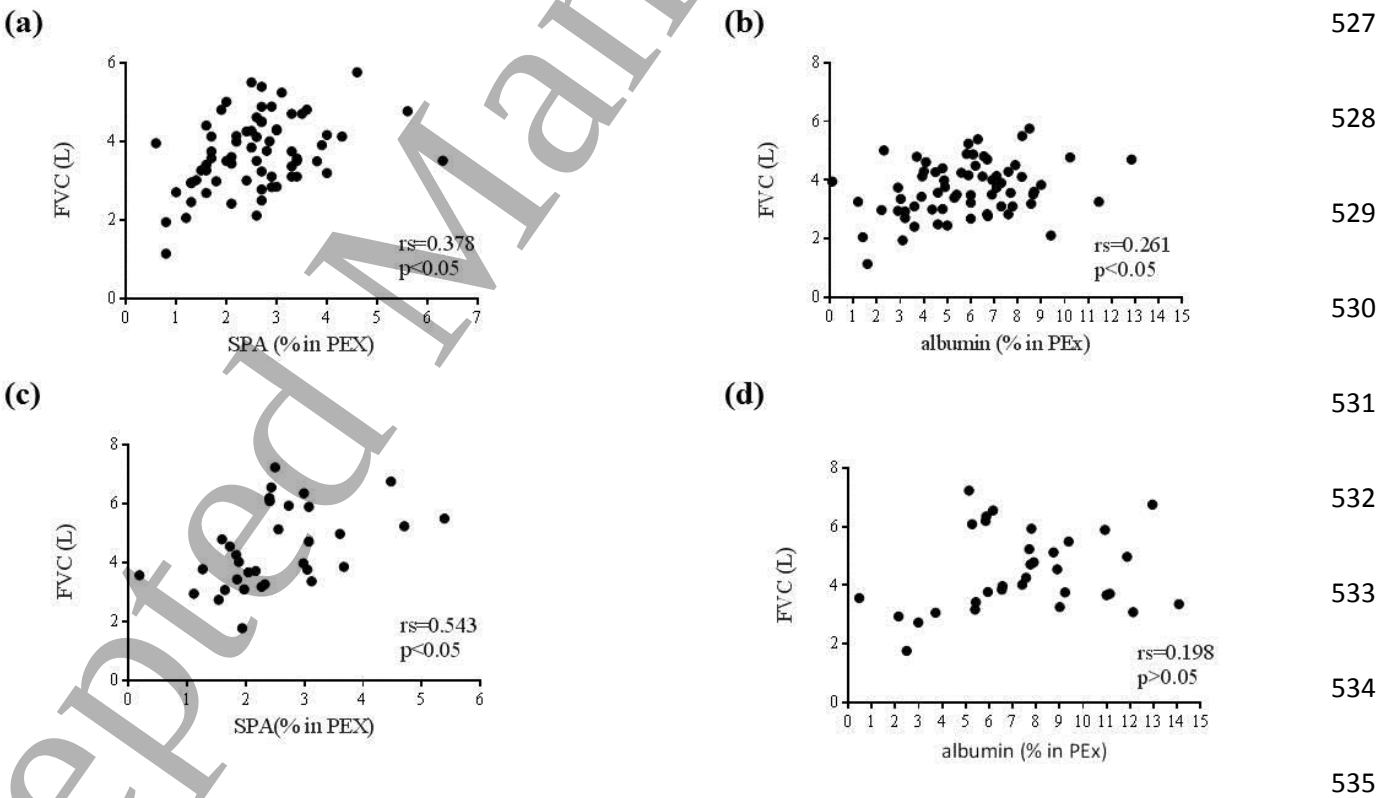


Figure 4 a, b, c, d: PEX and FVC in the replication cohort. Correlation between absolute FVC (Forced Vital Capacity) and %SPA and % of albumin, in the discovery population (4a, 4b) and replication population (4c, 4d).

539 Discussion

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

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

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

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

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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 mechanism 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).

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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.

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