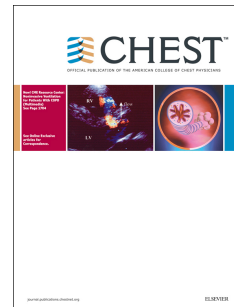


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CT and functional MRI to evaluate airway mucus in severe asthma

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Abstract: 236
Main text: 3317
References: 41

CT and functional MRI to evaluate airway mucus in severe asthma

Running Head: Luminal contributors to ventilation heterogeneity

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ABBREVIATIONS LIST

45	
46	
47	ACQ: asthma control questionnaire
48	AQLQ: asthma quality of life questionnaire
49	BD: bronchodilator
50	BMI: body mass index
51	CT: computed tomography
52	EB: eosinophilic bronchitis
53	FeNO: fractional exhaled nitric oxide
54	FEV ₁ : forced expiratory volume in one second
55	FVC: forced vital capacity
56	ICS: inhaled corticosteroid
57	IL-4: interleukin 4
58	IL-5: interleukin 5
59	IL-13: interleukin 13
60	LABA: long acting beta agonist
61	LLL: left lower lobe
62	LUL: left upper lobe
63	MRI: magnetic resonance imaging
64	MS: mucus score
65	N.D.: not-determined
66	OCS: oral corticosteroid
67	RLL: right lower lobe
68	RML: right middle lobe
69	RUL: right upper lobe
70	VDP: ventilation defect percent
71	WL: whole lung

ABSTRACT

Background: Intra-luminal contributor(s) to airflow obstruction in severe asthma are patient-specific and must be evaluated to personalize treatment. The occurrence and functional consequence of airway mucus in the presence or absence of airway eosinophils remains undetermined. Therefore, our objective was to understand the functional consequence of airway mucus in the presence or absence of eosinophils and to identify biomarkers of mucus-related airflow obstruction.

Methods: We quantified mucus plugs on computed tomography scans and evaluated their contribution to ventilation heterogeneity (using the magnetic resonance imaging (MRI) ventilation defect percent (VDP)) in 27 severe asthmatics. Patients were dichotomized based on sputum eosinophilia such that the relationship between mucus, eosinophilia and ventilation heterogeneity could be interrogated. Exhaled nitric oxide (FeNO) and related cytokines in sputum were measured.

Results: Mucus plugging was present in 100% of asthmatics with sputum eosinophils and 36% of those without sputum eosinophils ($p=0.0006$) and was correlated with MRI VDP pre ($r=0.68$, $p=0.0001$) and post-bronchodilator ($r=0.72$, $p<0.0001$). In a multivariable regression, both mucus and eosinophils contributed to the prediction of post-bronchodilator MRI VDP ($R^2=0.75$, $p<0.0001$). Asthmatics in whom the mucus score was high, had raised FeNO ($p=0.03$) and IL-4 ($p=0.02$). Mucus plugging correlated with FeNO ($r=0.63$, $p=0.005$).

Conclusions: Both airway eosinophils and mucus can contribute to ventilation heterogeneity in severe asthmatics. Patients in whom mucus is the dominant cause of airway obstruction have evidence of an upregulated IL-4/IL-13 pathway that could be identified by raised FeNO.

Abstract: 236

Main text: 3317

References: 41

94 **Key Words:** severe asthma, sputum eosinophils, mucus plugs, computed tomography, magnetic
95 resonance imaging

INTRODUCTION

Luminal obstruction by mucus and cells is a common feature of fatal asthma^{1,2} that has been recognized by pathologists for more than a century. An emphasis has been placed on the contribution of cells, predominantly the eosinophil, to asthma symptoms and severity and in turn the development of biomarkers and biologics targeting the cellular component of luminal obstruction. In contrast, the presence of airway mucus, and its influence on asthma symptoms and severity, is poorly understood and largely ignored in clinical practice. This may be due to the lack of measurements to quantify airway mucus or because therapies targeting mucus hypersecretion have not been available.

With the development of biologics directed at the inhibition of interleukin-13 (IL-13), a potent stimulator of airway mucus³, therapies that have mucoregulatory effects are beginning to emerge.⁴⁻⁷ Appropriate strategies to treat severe asthmatics with currently available monoclonal antibodies should depend on the dominant intra-luminal contributor(s) to airflow obstruction. Hence, in addition to sputum cytometry to quantify cells within the airway lumen, an objective means to quantify airway mucus is now required. Towards this goal, a visual computed tomography (CT) scoring system to quantify airway mucus plugging was developed by Dunican et al.⁸ Using this approach, mucus plugs were detected on CT in 58% of severe asthmatics.⁸ A subset of plugs persisted over time, and were associated with airflow obstruction, exacerbations and increased sputum eosinophils.⁸ While it seems logical to hypothesize that airway-blocking mucus plugs result in airflow obstruction, the functional relevance of mucus in the presence or absence of airway eosinophils remains undetermined.

The functional consequence of luminal obstruction can be regionally measured using inhaled gas magnetic resonance imaging (MRI).⁹⁻²⁰ Heterogenous ventilation is observed on MR

Abstract: 236

Main text: 3317

References: 41

images of severe asthmatics and has been correlated with disease severity^{16,19}, exacerbations²¹ and identified as an independent and a better predictor of asthma control than the forced expiratory volume in one second (FEV₁) which is the traditional measurement of luminal obstruction.¹⁷ We recently ascertained the contribution of airway eosinophils to ventilation heterogeneity in patients with severe asthma.²² In this study, our objective was to understand the functional relevance of airway mucus in the presence or absence of airway eosinophils. We therefore quantified airway mucus (using CT) and ventilation heterogeneity (using MRI) in asthmatics with uncontrolled and controlled eosinophilic bronchitis (EB). Using sputum cytometry and CT we classified asthmatics based on the contributors to luminal plugging to better understand the independent or combined influence of mucus and eosinophils on ventilation. In order to identify clinical biomarkers of mucus-obstructing airways, we measured fractional exhaled nitric oxide (FeNO) and relevant cytokines (IL-4 and IL-13) in the cell-soluble fraction of processed sputum (referred hereafter as supernatant). Clinical integration of an objective multi-measurement approach to characterize airway mucus and eosinophils, as presented in this study, may help guide selection of adjunct therapy for individual patients with severe asthma.

MATERIALS AND METHODS

Participants and design

Adults with a diagnosis of severe asthma, according to the Global Initiative of Asthma treatment step criteria (16), and whose compliance was regularly checked (by self reports, pharmacy refills and serum cortisol levels), were recruited from the Firestone Institute for

Respiratory Health, McMaster University. The study was approved by the University of Western Ontario Health Sciences Research Ethics Board (No. 103516 & 104200) and written consent was obtained from all participants. During a study visit, participants completed the Asthma Control Questionnaire (ACQ) and Asthma Quality of Life Questionnaire (AQLQ) and underwent spirometry, plethysmography, hyperpolarized ^3He MRI (data reported in a prior publication²²) and thoracic CT at Robarts Research Institute, Western University. Spirometry, plethysmography and MRI were completed pre- and post-bronchodilator, while CT was only completed post-bronchodilator. As previously described²², participants were dichotomized according to the presence of eosinophilic bronchitis (EB).

Airway eosinophils

Airway eosinophils were quantified from sputum that was collected clinically²³ at the Firestone Institute for Respiratory Health (median time between sputum induction and study visit was 4 (0-283) days). As previously described²², patients were dichotomized according to the presence of sputum granulocytes: 1) uncontrolled EB ($\geq 3\%$ eosinophils or eosinophil free granules, with or without $\geq 64\%$ neutrophils with an increased total cell count of ≥ 9.7 million cells/g), or 2) controlled EB ($< 3\%$ eosinophils without eosinophil free granules, and $< 64\%$ neutrophils with or without an increased total cell count of ≥ 9.7 million cells/g) at the time of study.

Spirometry, plethysmography, bronchodilator reversibility and FeNO

Spirometry and plethysmography were performed using a *MedGraphics Elite Series* plethysmograph (MedGraphics; St. Paul, USA). For post-bronchodilator measurements, four 100 μg doses of *Novo-Salbutamol*[®] HFA (Teva Novopharm Ltd.; Toronto, Canada) were delivered through a metered dose inhaler using an *AeroChamber Plus* spacer (Trudell Medical

International; London, Canada). Participants withheld short-acting β -agonists for 6 hours, long-acting β -agonists for 12 hours and long-acting muscarinic antagonists for 24 hours prior to their study visit. FeNO was measured using a *Niox Mino*[®] or *Niox Vero*[®] (Aerocrine; Solna, Sweden) portable eNO analyzer. FeNO was available for 18 patients and the median time between FeNO collection and the imaging study visit was 2 (0-23) days.

Sputum cytokines

The concentration of IL-4 and IL-13 in sputum supernatants was quantified using a multiplex array (Human High Sensitivity 14-Plex Discovery Assay; Eve Technologies, Calgary, Canada). Phosphorylated STAT6 in sputum supernatant was assessed using an enzyme-linked immunosorbent assay (Catalog No.LS-F4679; LSBio, Seattle, USA).

CT and MRI acquisition

¹H and hyperpolarized ³He MRI were acquired within five minutes of one another, pre- and post-bronchodilator, using a 3T MR system (General Electric Health Care; Milwaukee, USA).²⁴ Patients were instructed to inhale 1L of gas (N₂ for ¹H MRI and a hyperpolarized ³He/N₂ mixture for ³He MRI) from functional residual capacity, and 15 coronal slices were acquired under breath-hold conditions. Thoracic CT images were acquired within 30 minutes of the post-bronchodilator MRI using a 64-slice Lightspeed VCT scanner (General Electric Health Care; Milwaukee, USA).²⁵ Patients were instructed to inhale 1L of N₂ gas from functional residual capacity and CT acquisition was performed under breath-hold to match MRI acquisition conditions.

Image analysis to quantify mucus plugs

CT datasets were evaluated for the presence of mucus plugs, defined as complete occlusion of the airway, using a visual lung segment-based scoring system developed by

Abstract: 236

Main text: 3317

References: 41

Dunican et al.⁸ The 20 lung segments were visually inspected and scored for the presence (score of 1) or absence (score of 0) of ≥ 1 mucus plug(s). Individual lung segment scores were summed to generate a whole lung mucus score, ranging from 0 to 20, for each participant. In a similar manner, lobe specific lung segments were summed to generate lobar mucus scores for the right upper lobe (RUL), right middle lobe (RML), right lower lobe (RLL), left upper lobe (LUL) and left lower lobe (LLL). Two radiologists independently scored all de-identified CT datasets. If the individual whole lung mucus scores differed by <4 points, the scores of both raters were averaged. If the individual scores differed by ≥ 4 points, the cases were re-scored together by both raters to reach a consensus. The inter-rater agreement is provided in the supplemental material.

Image analysis to quantify ventilation heterogeneity

MRI analysis was performed to quantify ventilation heterogeneity using semi-automated segmentation and registration software as previously described.^{24,26,27} Whole lung MRI ventilation defect percent (VDP) was generated as the ventilation defect volume normalized to the thoracic cavity volume.²⁴ In a similar manner, lobar VDP (VDP_{RUL}, VDP_{RML}, VDP_{RLL}, VDP_{LUL} and VDP_{LLL}) was calculated by normalizing the lobe specific ventilation defect volume to the corresponding lobe volume.²⁷

Statistical methods

Data were tested for normality using the Shapiro-Wilk normality test and when data were not normal, non-parametric tests were performed. Unpaired t-tests and Mann-Whitney U-tests were performed to compare patient characteristics and imaging measurements in asthmatics with: 1) uncontrolled and controlled EB, and 2) high (≥ 4) and low mucus scores (<4). Univariate relationships were evaluated using Pearson and Spearman correlation coefficients. To determine

the independent contribution of mucus and eosinophils to ventilation heterogeneity, stepwise multivariable linear regression models were generated. Statistical analyses were performed using GraphPad Prism 7.00 (La Jolla, USA) and SPSS 23.00 (Armonk, USA), results were considered significant when the probability of making a Type I error was $<5\%$ ($p < 0.05$).

RESULTS

Demographics and clinical characteristics for 27 severe asthmatics, including 13 with uncontrolled EB and 14 with controlled EB, are provided in Table 1.

Mucus plugging in asthmatics with uncontrolled and controlled EB

CT evidence of mucus plugging was present in one or more lung segments in 100% (13/13) of asthmatics with uncontrolled EB and in 36% (5/14) of asthmatics with controlled EB. As shown in Table 2, asthmatics with uncontrolled EB (11.0 [2.5-15.5]) had significantly higher CT mucus scores than asthmatics with controlled EB (0.0 [0.0-17.0]; $p=0.0006$). We also observed that a higher CT mucus score was associated with a higher percentage of sputum eosinophils ($r=0.82$, $p=0.0001$), worse airflow obstruction (FEV_1/FVC : $r=-0.44$, $p=0.02$) and air-trapping ($FRC\%_{pred}$: 0.41, $p=0.04$; $RV\%_{pred}$: $r=0.68$, $p=0.0001$; RV/TLC %: $r=0.51$, $p=0.007$) (previously reported by Dunican et al⁸). Additional univariate relationships between the CT mucus score and traditional pulmonary function measurements are provided in e-Table 1.

Functional consequence of contributors to luminal plugging

Table 2 summarizes whole lung and lobar MRI ventilation heterogeneity, quantified as the VDP, for all asthmatics and those with uncontrolled and controlled EB. Figure 1 shows MRI and CT datasets for four representative asthmatics and demonstrates worse ventilation in

asthmatics with more mucus plugging. Within individual patients this relationship is upheld regionally in different lobes. The median VDP was 3.0 times higher pre-bronchodilator ($p=0.0008$) and 2.3 times higher post-bronchodilator ($p=0.0004$) for asthmatics with a high mucus score than those with a low mucus score (Figure 2). We also visually inspected co-registered MRI and CT datasets and observed a spatial relationship between mucus plugs and ventilation defects (Figure 3).

Univariate relationships between MRI VDP and the CT mucus score are shown in Figure 4 for the whole lung and in Table 3 for each lung lobe. Worse MRI ventilation pre- and post-bronchodilator were correlated with a higher CT mucus score for the whole lung (pre-bronchodilator: $r=0.68$, $p=0.0001$; post-bronchodilator: $r=0.72$, $p<0.0001$ (Figure 4A and B)) and in each lobe except for the right lower lobe (Table 3). In accordance with our previous results²², worse MRI ventilation was correlated with higher sputum eosinophils (pre-bronchodilator: $r=0.80$, $p=0.0004$; post-bronchodilator: $r=0.67$, $p=0.004$). Stepwise multivariable regressions were performed to evaluate the independent contribution of mucus plugs (CT mucus score) and eosinophils (percentage of sputum eosinophils) to ventilation heterogeneity (MRI VDP) pre and post-bronchodilator (Table 4). Airway mucus ($\beta=0.79$, $p<0.0001$), but not eosinophils, added to the prediction of pre-bronchodilator VDP ($R^2=0.62$, $p<0.0001$). Whereas both airway mucus ($\beta=0.65$, $p=0.001$) and eosinophils ($\beta=0.33$, $p=0.049$) added significantly to the prediction of post-bronchodilator VDP ($R^2=0.75$, $p<0.0001$).

Classification based on contributors to luminal plugging

According to the criteria presented in Figure 5A, asthmatics with uncontrolled and controlled EB ($\geq 3\%$ eosinophils= eos^+ ; $<3\%$ eosinophils= eos^-)^{28,29} were subdivided based on the presence of mucus plugging (mucus score $\geq 1=\text{mucus}^+$; mucus score $<1=\text{mucus}^-$) to better

understand the independent or combined influence of mucus and eosinophils on ventilation. All 13 patients with uncontrolled EB had mucus plugging (eos⁺mucus⁺: n=13). Of the remaining 14 patients with controlled EB, 5 had mucus plugging (eos⁻mucus⁺: n=5) and 9 did not (eos⁻mucus⁻: n=9).

The eos⁺mucus⁺, eos⁻mucus⁺ and eos⁻mucus⁻ groups were different with respect to their pre- (p=0.01, Figure 5B) and post-bronchodilator VDP (p=0.002, Figure 5C) but were not different with respect to their FEV₁ or clinical characteristics (e-Table 2). A listing of clinical characteristics for those patients in the eos⁻mucus⁺ group is provided in e-Table 3. Pre-bronchodilator VDP was significantly higher for eos⁺mucus⁺ asthmatics (10.8±5.9%) compared to eos⁻mucus⁻ asthmatics (3.8±2.8%; p=0.007). Post-bronchodilator VDP was significantly higher for eos⁺mucus⁺ asthmatics (6.2±3.1%, p=0.004) and eos⁻mucus⁺ asthmatics (4.0±0.5%, p=0.02) compared to eos⁻mucus⁻ asthmatics (2.5±1.1%).

Mucus score is associated with an upregulated IL-4/IL-13 pathway

To determine if mucus observed on CT may serve as a biomarker of an upregulated IL-4/IL-13 pathway we examined FeNO along with sputum IL-4 and IL-13 for asthmatics with high and low mucus scores. FeNO was correlated with the mucus score (r=0.63, p=0.005) and was significantly higher in asthmatics with a high mucus score than asthmatics with a low mucus score (p=0.03, Figure 6A). Increased IL-4 and IL-13 (above 90th percentile of healthy population²⁸) were observed in sputum supernatants of 43% and 86% of asthmatics respectively. 67% of asthmatics with a high mucus score had increased IL-4, compared to 25% of asthmatics with a low mucus score (p=0.09). 89% of asthmatics with a high mucus score had increased IL-13, compared to 83% of patients with a low mucus score (p>0.99). Sputum IL-4 (p=0.02, Figure 6B), but not IL-13 (p=0.50, Figure 6C), was significantly higher in asthmatics with a high mucus

score than asthmatics with a low mucus score. Although a relationship between the CT mucus score and IL-13 was not evident in this dataset, IL-13 was correlated with FeNO ($r=0.45$, $p=0.05$).

DISCUSSION

We observed: 1) mucus plugs on CT for all asthmatics with EB and in $\sim 1/3$ of asthmatics without EB, 2) both airway eosinophils and mucus were associated with ventilation abnormalities identified by MRI, and 3) evidence of an upregulated IL-4/IL-13 pathway that was identified by raised FeNO in asthmatics in whom mucus is an intra-luminal contributor to airflow obstruction.

The median mucus score for the asthmatics we studied was 3.0, and those with uncontrolled EB had more mucus plugging than those with controlled EB (11.0 versus 0.0). Compared to the asthmatics studied by Dunican et al, in which the median mucus score was 0.5, our cohort consisted of more severe (100% vs. 66%) and OCS dependent asthmatics (56% vs. 10%) with greater sputum eosinophilia (4% vs. 0.7%). Strong evidence supports the notion that eosinophil-driven oxidation stimulates airway mucus hypersecretion and plug formation in asthma.^{8,29} Therefore, we were not surprised that mucus plugging was more prevalent in the asthmatics we studied, in particular those with uncontrolled EB. However, plugs were also observed in $\sim 1/3$ of asthmatics with controlled EB. This finding agrees with murine studies³⁰, and suggests that plugs may occur in the absence of eosinophils and that a distinct mechanism may be responsible for their formation. To test this hypothesis, CT-directed bronchoscopy studies are required to collect plugs from asthmatics with and without EB such that their composition can be investigated.

While Dunican et al clearly demonstrated that mucus may be present in the airways of severe asthmatics, our study ascertained the functional relevance of mucus plugging in terms of ventilation heterogeneity assessed by MRI. To the best of our knowledge this is the first study to report a relationship between luminal plugging quantified on CT and MRI VDP. In addition, asthmatics with a high mucus score had worse MRI VDP than those with a low mucus score. If an airway lumen is plugged, it seems logical to assume distal airflow limitation and indeed we observed luminal plugs that were spatially related to ventilation defects. We acknowledge that this association is complex and may be influenced by additional technical and anatomical variables. For example, flow through collateral channels may provide an alternative pathway to ventilate distal lung units despite obstruction of a segmental or subsegmental airway. The compensatory potential of collateral ventilation is likely to be dependent on the presence of luminal plugs in neighbouring airways and the anatomical site of luminal obstruction – factors that were not probed by the mucus score. Subsequent studies are necessary to understand how co-localization of plugs and partial airway occlusion impact ventilation. Automated CT-MRI co-registration pipelines similar to that proposed by Guo et al will be necessary to facilitate such regional structure-function comparisons at the segmental and sub-segmental level.³¹

Our previous work confirmed that ventilation abnormalities persist following bronchodilator in asthmatics with airway eosinophilia.²² Importantly, residual ventilation abnormalities that were not responsive to bronchodilator therapy were correlated with eosinophils in induced sputum.²² Considering our previous work²², and the well-documented relationship between mucus production and airway eosinophilia^{8,29}, we confirmed the functional consequence of airway mucus in the presence or absence of eosinophils in two ways. First, in a multivariable model, both mucus and eosinophils contributed to the prediction of post-

Abstract: 236

Main text: 3317

References: 41

325 bronchodilator ventilation heterogeneity. Second,
326 classification of asthmatics based on the intra-luminal contributors to airflow obstruction
327 revealed that mucus plugging in the presence or absence of airway eosinophilia is associated
328 with ventilation abnormalities that persist post-bronchodilator. Taken together, this data
329 suggests that eosinophils and/or mucus plugs can contribute to ventilation abnormalities that
330 persist post-bronchodilator in severe asthma. The identification of mucus plugging as a major
331 determinant of VDP is important when considering the use of imaging in severe asthma
332 endotyping.

333 IL-4 and IL-13 play an important pathobiological role in asthma and have been shown to
334 stimulate mucus production.^{3,32-34} Elevated FeNO may be considered a good index of elevated
335 IL-4/IL-13 in the airway as epithelial NO production is promoted by IL-4/IL-13 via STAT6
336 signalling.^{35,36} Accordingly, we speculate that the presence of mucus in severe asthmatics may
337 be the result of a heightened IL-4/IL-13/STAT6 pathway of which FeNO may be a clinical
338 biomarker. While there is convincing evidence of the role of IL-13 in mucus production in
339 asthma³⁷, a relationship between mucus and IL-13 was not evident in our dataset. Dunican et al
340 reported that IL-13 and the ratio of MUC5AC to MUC5B gene expression in sputum cells was
341 higher in asthmatics with a high mucus score, however, MUC5AC expression was not altered by
342 anti-IL-13 therapy in the MESOS trial of tralokinumab³⁸. IL-4 on its own is also capable of
343 inducing goblet cell hyperplasia and mucus hypersecretion.³⁹ Perhaps there may be differences in
344 the in the quantities of IL-4 and IL-13 produced in non-bone marrow-derived cells, the
345 distribution of type II IL-4Rs in non-hematopoietic structural cells in the airways, and the low
346 abundance of IL-13R α relative to that of IL-4R α at the site of inflammation.⁴⁰ We are unable to
347 comment on STAT6 as the concentration in sputum supernatants was below the detectable range

Abstract: 236

Main text: 3317

References: 41

(data not shown). Regardless, patients in whom mucus is a contributor to airway obstruction have evidence of an upregulated IL-4/IL-13 pathway that could be identified by raised FeNO, making them potential candidates for anti-IL-4/IL-13 therapy (eg. Dupilumab^{4,41}). Prospective studies are warranted to answer the following: Are mucus plugs responsive to anti-IL-4/IL-13 therapy? Does clearance of mucus restore ventilation? Can mucus plugging on CT and/or elevated FeNO be used to select patients who benefit from anti-IL-4/IL-13 therapy?

The strengths of this study include the fact that patients were well-characterized with severe disease and that imaging was timed to coincide with clinical evaluations. We also recognize study limitations. We used a relatively crude visual scoring system to quantify mucus plugs on CT⁸ due to challenges associated with automated segmentation methods. The mucus score is not specific to luminal mucin, but instead reflects luminal plugs that may be composed of mucins, fibrin, and/or inflammatory cells. We also acknowledge that additional factors not probed by the mucus score may be relevant to the relationship between plugs and ventilation (eg. plug size, anatomical location and number of airways involved within each bronchopulmonary segment). Furthermore, the presence of partially occluded airways by mucus and/or remodelling is likely to influence ventilation but was not considered. Although a study shortcoming, the mucus score is physiologically relevant and can be easily implemented into clinical workflow. Finally, we did not repeat the MRI or CT and therefore cannot comment on the repeatability of ventilation defects or mucus plugs in these patients. Studies are now ongoing to evaluate the effect of T2 directed therapies on ventilation defects and mucus plugs.

CONCLUSIONS

Abstract: 236

Main text: 3317

References: 41

371 Both airway eosinophils and mucus can contribute to ventilation abnormalities that
372 persist post-bronchodilator in severe asthmatics. Asthmatics in whom mucus is a major
373 contributor to airway obstruction have evidence of an upregulated IL-4/IL-13 pathway that could
374 be identified by raised FeNO. Accurate endotyping to identify patients in whom excess mucus
375 production is the dominant contributor to airflow obstruction may elicit greater clinical effect of
376 anti-IL-4/IL-13 therapy whereas those with dominant eosinophil-related airflow obstruction may
377 benefit from anti-IL5 therapy.

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Author Contributions: S.S. was responsible for CT and MRI data collection, analysis and interpretation, and for preparing the first and all revised drafts of the manuscript. E.H. and C.B. were responsible for reviewing all CT datasets to generate the CT mucus score and for the interpretation of CT data. M.M. was responsible for sputum cytokine analysis and interpretation. R.L.E. and D.P.I.C. were responsible for MRI data acquisition and analysis. G.P. was responsible for MRI and CT image acquisition, analysis and interpretation as well as guarantor of integrity of the imaging data. P.N. was responsible for conception and study design, identifying and characterizing the patients, for clinical interpretation of the data as well as guarantor of integrity of the clinical data. All authors edited and reviewed the manuscript and approved the final version of the manuscript.

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TABLES

Table 1. Patient demographics and clinical characteristics

	All (n=27)	Uncontrolled EB (n=13)	Controlled EB (n=14)	Significance of difference (p)*
Age yrs	46±14	51±13	42±14	0.13
Female sex n (%)	20 (74)	9 (69)	11 (79)	N.D.
BMI kg/m ²	29±5	29±5	28±4	0.51
ACQ Score	1.8±1.0	1.8±1.1	1.7±0.9	0.84
AQLQ Score	5.1±1.3	5.0±1.2	5.2±1.4	0.71
FEV ₁ Pre-BD % _{pred}	69±21 ^{††}	67±18 [‡]	70±23	0.74
FEV ₁ Post-BD % _{pred}	80±17	78±15	81±20	0.70
FVC Pre-BD % _{pred}	84±17 ^{††}	84±13 [‡]	84±20	0.90
FVC Post-BD % _{pred}	90±13	88±9	91±16	0.68
Reversibility of FEV ₁ % [†]	14 (0-99) ^{††}	15 (0-70) [‡]	11.5 (1-99)	0.53
Reversibility of FVC % [†]	5 (-10-62) ^{††}	6 (-7-29) [‡]	4.5 (-10-62)	0.94
Sputum eosinophils % [†]	4.0 (0.0-56.8) ^{‡‡}	9.2 (3.3-56.8) [§]	0.8 (0.0-2.5) [‡]	N.D.
FeNO ppb [†]	18 (5-146) ^{§§}	24 (11-83) [§]	12 (5-146) ^{**}	0.03
Nasal polyps n (%)	10 (37)	6 (46)	4 (29)	N.D.
Atopic n (%)	25 (93)	11 (85)	14 (100)	N.D.
Asthma Medications				
LABA dose µg/day [†]	100 (50-200)	100 (50-200)	100 (83-150)	0.98
ICS dose µg/day [†]	1400(400-4000)	2000(400-4000)	1200(400-2000)	0.05
OCS dose mg/day [†]	5 (0-30)	7.5 (0.0-30.0)	0.0 (0.0-10.0)	0.002
OCS dependent n (%)	15 (56)	11 (85)	4 (29)	N.D.
Monoclonal antibody n (%)	9 (33)	2 (15)	7 (50)	N.D.
Omalizumab n	5	1	4	N.D.
Mepolizumab n	1	1	0	N.D.
Benralizumab n	1	0	1	N.D.
Dupilumab n	2	0	2	N.D.

Values are mean ± standard deviation except when indicated otherwise.

EB=eosinophilic bronchitis; N.D.=not-determined; BMI=body mass index; ACQ=asthma control questionnaire; AQLQ=asthma quality of life questionnaire; FEV₁=forced expiratory volume in one second; BD=bronchodilator; FVC=forced vital capacity; FeNO=fractional exhaled nitric oxide; LABA=long acting beta agonist; ICS=inhaled corticosteroid; OCS=oral corticosteroid.

*Significance of difference (p<0.05) between groups determined using an unpaired t-test for parametric data or Mann-Whitney test for nonparametric data; [†]Median (range); [‡]n=12; [§]n=10; ^{‡‡}n=7; ^{**}n=8; ^{††}n=26; ^{‡‡}n=17; ^{§§}n=18.

Table 2. Whole lung and lobar CT and MRI measurements

	All (n=27)	Uncontrolled EB (n=13)	Controlled EB (n=14)	Significance of difference (p)*
CT mucus plug score				
Post-BD whole lung [†]	3.0 (0.0-17.0)	11.0 (2.5-15.5)	0.0 (0.0-17.0)	0.0006
Post-BD RUL [†]	0.0 (0.0-3.0)	1.0 (0.0-3.0)	0.0 (0.0-2.0)	0.01
Post-BD RML [†]	0.5 (0.0-2.0)	1.0 (0.0-2.0)	0.0 (0.0-2.0)	0.02
Post-BD RLL [†]	1.0 (0.0-5.0)	2.0 (0.0-5.0)	0.0 (0.0-4.0)	0.003
Post-BD LUL [†]	0.5 (0.0-5.0)	3.0 (0.5-5.0)	0.0 (0.0-5.0)	<0.0001
Post-BD LLL [†]	0.5 (0.0-5.0)	3.0 (0.0-5.0)	0.0 (0.0-5.0)	0.006
MRI ventilation defect percent (%)				
Pre-BD whole lung	8.1±5.8 [‡]	10.8±5.9 [‡]	5.9±4.8	0.02
Pre-BD RUL	5.4±7.2 ^{**}	6.7±7.5 [‡]	4.0±7.0 [§]	0.12
Pre-BD RML	9.8±10.1 ^{**}	11.4±10.0 [‡]	8.2±10.4 [§]	0.24
Pre-BD RLL	10.1±8.4 ^{**}	13.2±10.0 [‡]	7.9±4.7 [§]	0.21
Pre-BD LUL	5.9±5.1 ^{**}	6.5±3.0 [‡]	5.2±6.9 [§]	0.09
Pre-BD LLL	12.9±10.3 ^{**}	16.4±11.9 [‡]	9.1±6.9 [§]	0.17
Post-BD whole lung	4.6±2.8	6.2±3.1	3.1±1.2	0.004
Post-BD RUL	3.0±4.3 ^{**}	4.7±5.3 [‡]	1.1±1.3 [§]	0.006
Post-BD RML	3.5±3.5 ^{**}	5.3±3.7 [‡]	1.6±1.9 [§]	0.007
Post-BD RLL	6.3±4.0 ^{**}	7.9±4.5 [‡]	4.6±2.8 [§]	0.045
Post-BD LUL	3.3±3.0 ^{**}	4.9±3.2 [‡]	1.4±1.3 [§]	0.006
Post-BD LLL	6.3±5.5 ^{**}	8.4±5.9 [‡]	4.1±2.4 [§]	0.04

Values are mean ± standard deviation except when indicated otherwise.

EB=eosinophilic bronchitis; CT=computed tomography; RUL=right upper lobe; RML=right middle lobe; RLL=right lower lobe; LUL=left upper lobe; LLL=left lower lobe; BD=bronchodilator.

*Significance of difference (p<0.05) between groups determined using an unpaired t-test for parametric data or Mann-Whitney test for nonparametric data; [†]Median (range); [‡]n=12; [§]n=11; [‡]n=26; ^{**}n=23.

Abstract: 236

Main text: 3317

References: 41

531 **Table 3.** Univariate relationships between CT mucus score and MRI ventilation defect percent
 532 for the whole lung and individual lobes

	Spearman r (p)	
	Pre-bronchodilator	Post-bronchodilator
Whole lung	0.68 (0.0001)*	0.72 (<0.0001)
Right upper lobe	0.48 (0.02) [†]	0.42 (0.046) [†]
Right middle lobe	0.46 (0.03) [†]	0.44 (0.04) [†]
Right lower lobe	0.53 (0.01) [†]	0.33 (0.12) [†]
Left upper lobe	0.62 (0.002) [†]	0.69 (0.0002) [†]
Left lower lobe	0.70 (0.0002) [†]	0.60 (0.003) [†]

533 *n=26, [†]n=23

Table 4. Multivariable linear regression models to predict MRI VDP

	Unstandardized		Standardized	P-value
Variable	B	Standard Error	β	
Pre-bronchodilator MRI VDP ($R=0.79$; $R^2=0.62$, $p<0.0001$)				
CT mucus score	0.82	0.17	0.79	<0.0001
Sputum eosinophils %	NA	NA	NA	NA
Post-bronchodilator MRI VDP ($R=0.87$; $R^2=0.75$, $p<0.0001$)				
CT mucus score	0.36	0.09	0.65	0.001
Sputum eosinophils %	0.08	0.04	0.33	0.049

Bold values denote statistical significance. B=unstandardized regression coefficient; β =standardized regression coefficient; NA=not added to model.

FIGURE LEGENDS**Figure 1. CT mucus scores and MRI ventilation heterogeneity for representative severe asthmatics**

Centre coronal slice MRI ventilation in cyan co-registered to anatomical ^1H MRI in grey-scale for four representative severe asthmatics demonstrating worse ventilation heterogeneity in patients with mucus plugging identified on CT. CT mucus score (MS) and MRI ventilation defect percent (VDP) pre- and post-bronchodilator are provided for the whole lung (WL), right upper lobe (RUL), right middle lobe (RML), right lower lobe (RLL), left upper lobe (LUL) and left lower lobe (LLL).

Subject 09, mucus score=0.0: 45 year-old female, 0.5% sputum eosinophils, ACQ=1.6, pre-BD $\text{FEV}_1=103\%_{\text{pred}}$, post-BD $\text{FEV}_1=115\%_{\text{pred}}$, pre-BD VDP=3%, post-BD VDP=2%.

Subject 35, mucus score=3.0: 53 year-old female, 3.3% sputum eosinophils, ACQ=1.4, pre-BD $\text{FEV}_1=56\%_{\text{pred}}$, post-BD $\text{FEV}_1=74\%_{\text{pred}}$, pre-BD VDP=8%, post-BD VDP=3%.

Subject 38, mucus score=10.0: 69 year-old male, 5.0% sputum eosinophils, ACQ=0.6, pre-BD $\text{FEV}_1=101\%_{\text{pred}}$, post-BD $\text{FEV}_1=107\%_{\text{pred}}$, pre-BD VDP=16%, post-BD VDP=8%.

Subject 30, mucus score=12.0: 45 year-old female, 56.8% sputum eosinophils, ACQ=1.5, pre-BD $\text{FEV}_1=46\%_{\text{pred}}$, post-BD $\text{FEV}_1=60\%_{\text{pred}}$, pre-BD VDP=17%, post-BD VDP=11%.

Figure 2. Worse MRI ventilation heterogeneity in asthmatics with high CT mucus score

(A) Pre-bronchodilator MRI VDP was statistically significantly higher for asthmatics with a high CT mucus score compared to asthmatics with a low CT mucus score ($p=0.0008$).

(B) Post-bronchodilator MRI VDP was statistically significantly higher for asthmatics with a high CT mucus score compared to asthmatics with a low CT mucus score ($p=0.0004$).

Box and whisker plots show the minimum, 25th percentile, median, 75th percentile and maximum with individual data points for all patients superimposed on the plot. P-values were determined using Mann-Whitney tests.

Figure 3. Spatial relationship between mucus plugs and ventilation defects for two representative asthmatics

Yellow crosshairs identify the spatial relationship between a focal mucus plug observed on CT (window width, 1200 HU; window level, -600 HU) and ventilation defect observed on MRI (ventilation MRI in cyan co-registered to anatomical ¹H MRI in grey-scale). The left most panel shows the mucus plug in the axial, sagittal and coronal view.

Subject 37, mucus plug in the RB1 segment: 58 year-old female, 15.8% sputum eosinophils, mucus score=13.5; ACQ=1.0, pre-BD FEV₁=81%_{pred}, post-BD FEV₁=91%_{pred}, pre-BD VDP=14%, post-BD VDP=9%.

Subject 39, mucus plug in the LB7 segment: 57 year-old male, 13.3% sputum eosinophils, mucus score=14.0; ACQ=1.9, pre-BD FEV₁=79%_{pred}, post-BD FEV₁=86%_{pred}, pre-BD VDP=15%, post-BD VDP=10%.

Figure 4. Univariate relationships between the CT mucus score and MRI ventilation defect percent

(A) CT mucus plug score was significantly correlated with pre-bronchodilator MRI VDP ($r=0.68$, $p=0.0001$).

(B) CT mucus plug score was significantly correlated with post-bronchodilator MRI VDP ($r=0.72$, $p<0.0001$).

Dotted lines=95% confidence intervals.

Figure 5. Classification of patients based on the contributors (mucus and sputum eosinophils) to luminal plugging

(A) Patients were divided into four groups based on the presence of sputum eosinophils ($\geq 3\%$ sputum eosinophils= eos^+ ; $<3\%$ sputum eosinophils= eos^-) and mucus plugging (CT mucus score ≥ 1 =mucus $^+$; CT mucus score <1 =mucus $^-$): 1) $\text{eos}^+\text{mucus}^+$, n=13; 2) $\text{eos}^-\text{mucus}^+$, n=5; 3) $\text{eos}^+\text{mucus}^-$, n=0 and 4) $\text{eos}^-\text{mucus}^-$, n=9.

(B) There was a statistically significant difference in pre-bronchodilator VDP between groups ($p=0.01$). Pre-bronchodilator VDP was statistically significantly higher for $\text{eos}^+\text{mucus}^+$ patients ($10.8 \pm 5.9\%$) compared to $\text{eos}^-\text{mucus}^-$ patients ($3.8 \pm 2.8\%$) ($p=0.007$). There was no statistically significant difference between $\text{eos}^+\text{mucus}^+$ and $\text{eos}^-\text{mucus}^+$ patients ($p=0.94$) or $\text{eos}^+\text{mucus}^+$ and $\text{eos}^-\text{mucus}^-$ patients ($p=0.17$).

(C) There was a statistically significant difference in post-bronchodilator VDP between groups ($p=0.002$). Post-bronchodilator VDP was significantly higher for $\text{eos}^+\text{mucus}^+$ patients ($6.2 \pm 3.1\%$, $p=0.004$) and $\text{eos}^-\text{mucus}^+$ patients ($4.0 \pm 0.5\%$, $p=0.02$) compared to $\text{eos}^-\text{mucus}^-$ patients ($2.5 \pm 1.1\%$). There was no statistically significant difference between $\text{eos}^+\text{mucus}^+$ and $\text{eos}^-\text{mucus}^+$ patients ($p=0.07$).

Box and whisker plots show the minimum, 25th percentile, median, 75th percentile and maximum with individual data points for all patients superimposed on the plot.

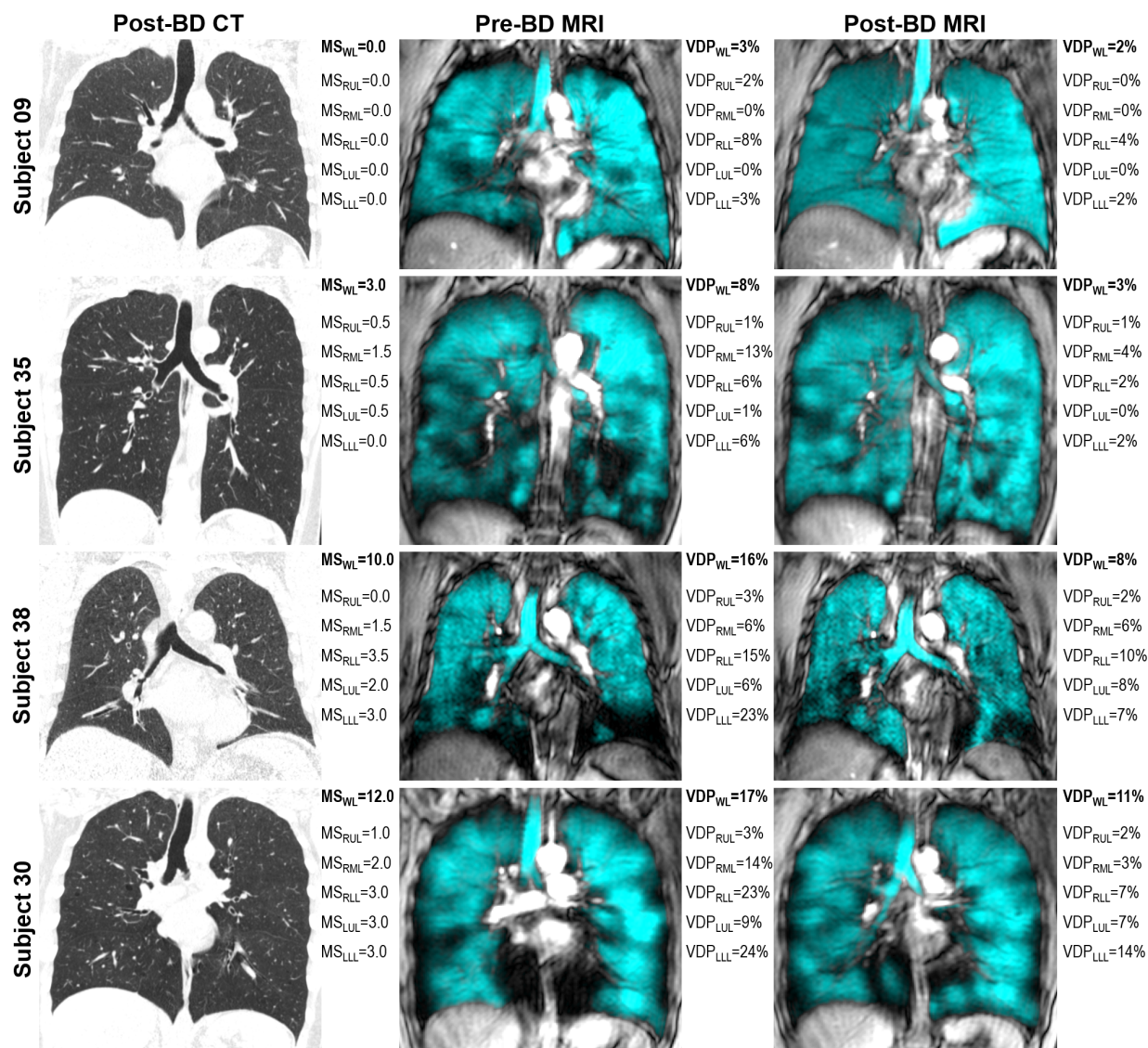
Figure 6. CT mucus score is associated with IL-4/IL-13 pathway

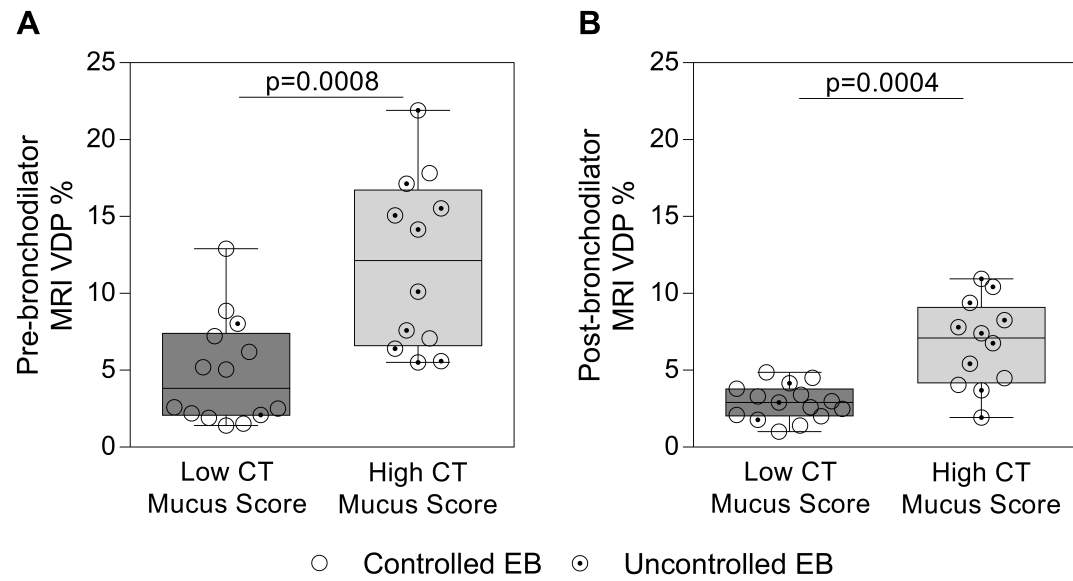
(A) FeNO was statistically significantly higher for asthmatics with a high CT mucus score compared to asthmatics with a low CT mucus score ($p=0.03$).

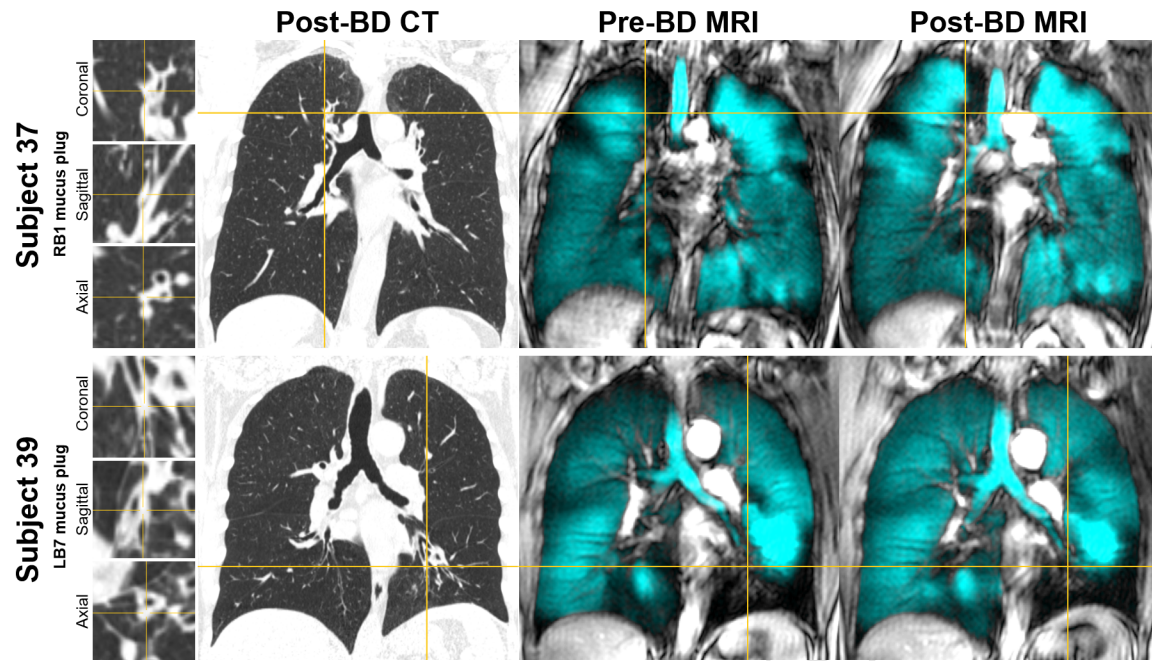
(B) Sputum IL-4 was statistically significantly higher for asthmatics with a high CT mucus score compared to asthmatics with a low CT mucus score ($p=0.02$). The dotted line represents the 90th percentile threshold of healthy.

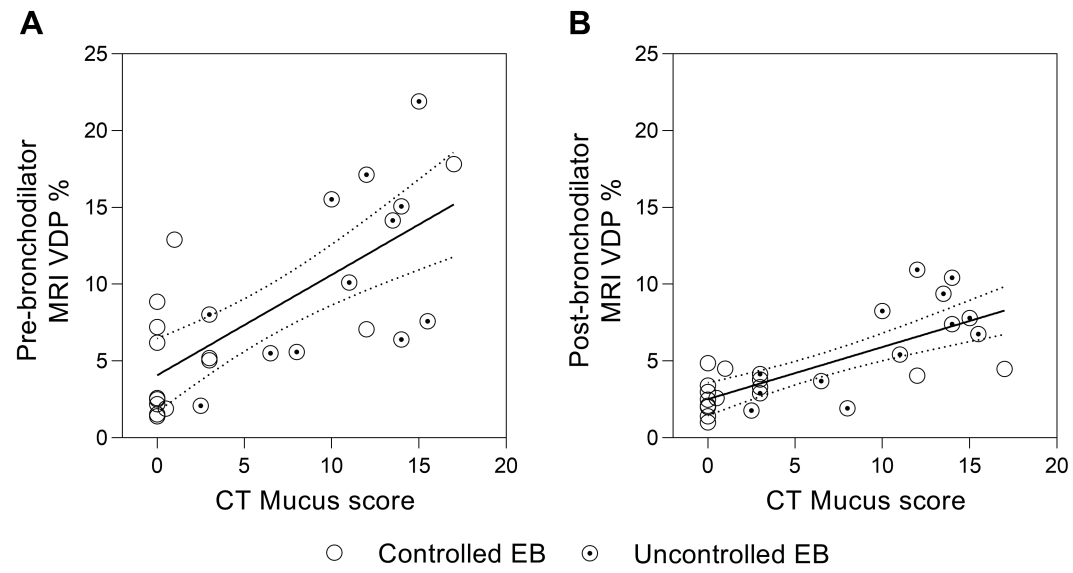
(C) Sputum IL-13 was not significantly different for asthmatics with high and low CT mucus scores ($p=0.50$). The dotted line represents the 90th percentile threshold of healthy.

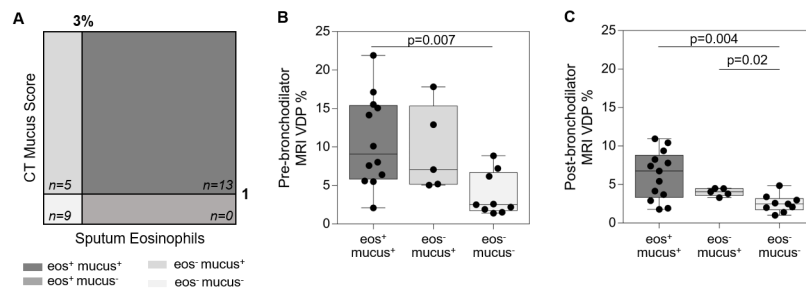
Box and whisker plots show the minimum, 25th percentile, median, 75th percentile and maximum with individual data points for all patients superimposed on the plot. P-values were determined using Mann-Whitney tests.

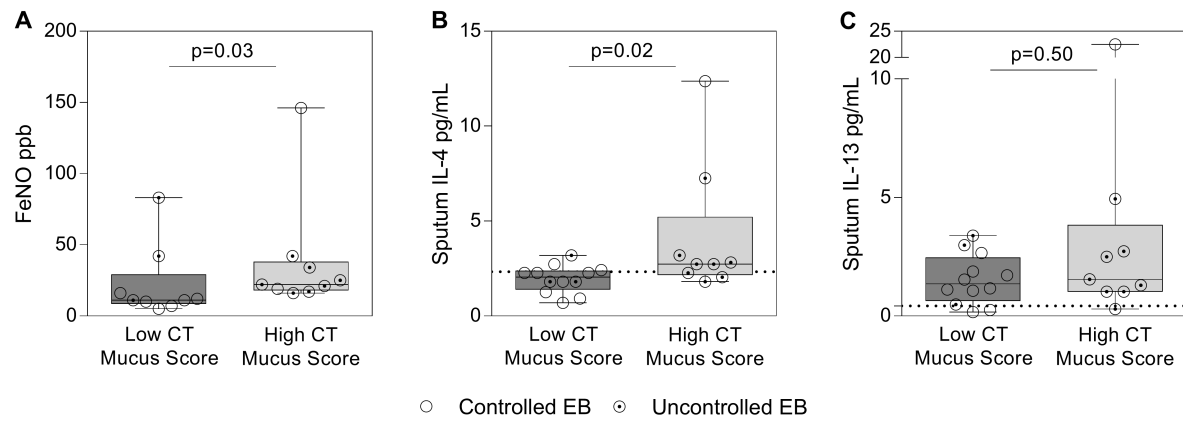












e-Appendix 1.

CT mucus score inter-rater agreement: Two radiologists, who were blinded to clinical data and study measurements, independently scored all de-identified CT datasets. The inter-rater bias and agreement of the CT mucus score were evaluated by a wilcoxon matched-pairs signed rank test and intraclass correlation coefficient (ICC), respectively. ICC estimates and their 95% confident intervals were calculated using SPSS 23.00 (Armonk, USA) based on a mean-rating ($k = 2$), absolute-agreement, 2-way random-effects model. For all 27 CT datasets, there was no statistically significant bias between the raters (mucus scores: rater 1= 6.0 ± 6.5 , rater 2= 3.5 ± 4.5 , $p=0.09$) and the ICC was 0.65 (95% confidence interval 0.26 to 0.84). To compensate for poor agreement in a subset of datasets, if the individual mucus scores differed by <4 points, the scores of both raters were averaged. If the individual scores differed by ≥ 4 points, the cases were re-scored together by both raters to reach a consensus. The inter-rater difference in the CT mucus score was ≥ 4 points for 9 of 27 datasets. **The discordant assessments of the mucus plugging by the radiologists was due to a consistent difference between each radiologist in interpreting the appearance of sub-segmental airways with mucus plugging, affecting sensitivity for mucus plugging and associated scoring. This systematic interpretative difference was easily resolved with discussion. This allowed for more uniform scoring prospectively when reinterpreting cases.** For the 18 CT datasets that differed by <4 points, there was no statistically significant bias between the raters (mucus scores: rater 1= 3.0 ± 4.5 , rater 2= 3.5 ± 5.5 , $p=0.20$) and the ICC was 0.97 (95% confidence interval 0.92 to 0.99). Intra-rater agreement was not evaluated.

e-Table 1. Univariate relationships between the CT mucus score, MRI VDP and traditional measurements of lung function

	Spearman r (p)		
	CT Mucus Score	MRI VDP	
	Post-BD	Pre-BD	Post-BD
<i>Spirometry</i>			
FEV ₁ Pre-BD % _{pred}	-0.29, 0.15	-0.46, 0.02	-0.21, 0.29
FEV ₁ Post-BD % _{pred}	-0.26, 0.19	-0.35, 0.08	-0.18, 0.36
FVC Pre-BD % _{pred}	-0.27, 0.18	-0.30, 0.14	-0.15, 0.46
FVC Post-BD % _{pred}	-0.22, 0.28	0.02, 0.91	-0.03, 0.87
FEV ₁ /FVC Pre-BD %	-0.42, 0.03	-0.67, 0.0002	-0.39, 0.05
FEV ₁ /FVC Post-BD %	-0.44, 0.02	-0.72, <0.0001	-0.49, 0.01
<i>Plethysmography</i>			
FRC Pre-BD % _{pred}	0.41, 0.04	0.62, 0.0008	0.47, 0.02
FRC Post-BD % _{pred}	0.41, 0.04	0.61, 0.001	0.52, 0.006
RV Pre-BD % _{pred}	0.37, 0.06	0.66, 0.0002	0.39, 0.05
RV Post-BD % _{pred}	0.68, 0.0001	0.62, 0.001	0.53, 0.005
TLC Pre-BD % _{pred}	0.29, 0.15	0.42, 0.03	0.31, 0.12
TLC Post-BD % _{pred}	0.30, 0.14	0.48, 0.02	0.38, 0.06
RV/TLC Pre-BD %	0.55, 0.003	0.64, 0.0004	0.53, 0.006
RV/TLC Post-BD %	0.51, 0.007	0.50, 0.01	0.54, 0.004

Bold values denote statistically significant relationships.

FEV₁=forced expiratory volume in one second; BD=bronchodilator; FVC=forced vital capacity;
FRC=functional residual capacity; RV=residual volume; TLC=total lung capacity.

e-Table 2. Demographics and clinical characteristics for patients classified based on the contributors (sputum eosinophils and mucus) to luminal plugging.

	Mucus⁺		Mucus⁻	Significance of difference (p)	
	Eos⁺ (n=13)	Eos⁻ (n=5)	Eos⁻ (n=9)	All groups*	Eos+ Mucus+ vs. Eos-Mucus+ [†]
Age yrs	51±13	42±12	42±15	0.32	0.25
Female sex n (%)	9 (69)	4 (80)	7 (78)	--	--
BMI kg/m ²	29±5	30±5	27±4	0.55	0.91
ACQ Score	1.8±1.1	1.9±1.4	1.7±0.6	0.93	0.95
AQLQ Score	5.0±1.2	5.3±1.7	5.1±1.3	0.91	0.69
FEV ₁ Pre-BD % _{pred}	67±18 [§]	54±20	79±21	0.09	0.21
FEV ₁ Post-BD % _{pred}	78±15	68±14	88±20	0.09	0.18
FVC Pre-BD % _{pred}	84±13 [§]	76±18	88±21	0.39	0.32
FVC Post-BD % _{pred}	88±9	89±7	92±20	0.41	0.95
Reversibility of FEV ₁ % [‡]	15(0-70) [§]	11(6-99)	12 (1-28)	0.61	0.96
Reversibility of FVC % [‡]	6(-7-29) [§]	14(-10-62)	1 (-3-22)	0.13	0.13
FeNO ppb	24(11-83) ^{††}	19(7-146)	11 (5-16) ^{**}	0.02	0.79
Asthma Medications					
LABA dose µg/day [‡]	100(50-200)	100(83-100)	100(100-150)	0.55	0.65
ICS dose µg/day [‡]	2000(400-4000)	1400(500-2000)	1000(400-2000)	0.13	0.34
OCS dose mg/day [‡]	7.5(0.0-30.0)	0.0(0.0-5.0)	0.0(0.0-10.0)	0.008	0.02
OCS dependent n (%)	11(85)	2(40)	2 (22)	--	--

Values are mean ± standard deviation except when indicated otherwise.

BMI=body mass index; ACQ=asthma control questionnaire; AQLQ=asthma quality of life questionnaire; FEV₁=forced expiratory volume in one second; BD=bronchodilator; FVC=forced vital capacity; FeNO=fractional exhaled nitric oxide; LABA=long acting beta agonist; ICS=inhaled corticosteroid; OCS=oral corticosteroid.

*Significance of difference between groups determined using an ANOVA for parametric data or Independent-samples Kruskal Wallis Test for nonparametric data; [†]Significance of difference between groups determined using a t-test for parametric data or Independent-samples Mann-Whitney U test for nonparametric data; [‡]Median (range); [§]n=12; ^{||}n=3; ^{**}n=5; ^{††}n=10.

e-Table 3. Clinical characteristics for eos⁻mucus⁺ patients

	Subject 017	Subject 014	Subject 040	Subject 010	Subject 006
Mucus plug score	17	12	3	3	1
Sputum eosinophil %	IS	IS	IS	IS	1.5
Sputum EFG	IS	IS	IS	IS	None
Hx of sputum eosinophilia	(-)	(-)	(+)	(+)	(-)
FeNO ppb	146	19	7	42	--
FEV ₁ Pre-BD % _{pred}	34	55	73	75	33
Reversibility of FEV ₁ %	99	6	8	11	51
Blood eosinophils	0.5	0.4	0.1	0.0	0.1
ACQ	3.6	2.9	0.4	0.4	2.0
AQLQ	2.9	4.0	6.8	6.8	5.8

IS=patient was unable to provide a sufficient sputum sample (<0.08g of sputum separated from saliva) following three rounds of saline inhalation; EFG=eosinophil free granules; Hx=history; FeNO= fractional exhaled nitric oxide; FEV₁=forced expiratory volume in one second; BD=bronchodilator; ACQ=asthma control questionnaire; AQLQ=asthma quality of life questionnaire.