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Type 2 Inflammation in Eosinophilic Chronic Obstructive Pulmonary Disease

To the Editor

Type 2 (T2) inflammation in asthma encompasses the presence of eosinophilic inflammation and / or selected cytokines including interleukin (IL)-13. Some chronic obstructive pulmonary disease (COPD) patients have increased airway eosinophil numbers (1), which is associated with greater effects of inhaled corticosteroids (ICS) (2). Studies of T2 inflammation in COPD have used hypothesis free, open platform approaches (3, 4). We used these results, plus data from T2 asthma studies (5), to test a restricted panel of biomarkers (chloride channel accessory 1 [CLCA1], periostin [POSTN], serpin family B member 2 [SERPINB2], C-C motif chemokine ligand 26 [CCL26], IL-13 and cystatin SN [CST1]) to further investigate the nature of T2 inflammation in eosinophilic COPD.

We used two cohorts: bronchial brushings and sputum were obtained from 17 eosinophil^{high} and 20 eosinophil^{low} COPD patients (bronchoscopy cohort previously reported (1): eosinophil^{high} defined as blood eosinophils >250 eosinophils μL^{-1} and eosinophil^{low} defined as blood eosinophils <150 eosinophils μL^{-1}) and sputum only was obtained from 15 eosinophil^{high} and 18 eosinophil^{low} COPD patients (validation cohort: eosinophil^{high} defined as >150 eosinophils μL^{-1} blood and >3% sputum eosinophils and eosinophil^{low} defined as <150 eosinophils μL^{-1} blood and <3% sputum eosinophils). Full details of inclusion criteria and clinical characteristics are in the online supplement and table 1. Both cohorts were generally well matched. RNA sequencing examined

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gene expression in the bronchoscopy cohort, while RT-qPCR was used in the validation cohort. Full details of methods and analysis are in the online supplement.

In the bronchoscopy cohort, bronchial epithelial gene expression of CLCA1, CCL26, IL-13 and CST1 was significantly higher ($p < 0.05$) in eosinophil^{high} versus eosinophil^{low} COPD patients (figure 1) with fold change differences of 1.9, 2.2, 2.9 and 2.5 respectively. POSTN and SERPINB2 expression trended higher in eosinophil^{high} versus eosinophil^{low} COPD patients but did not achieve statistical significance ($p = 0.06$ and $p = 0.07$ respectively; supplementary figure S1).

In the bronchoscopy and validation cohorts, sputum gene expression of CLCA1, CCL26, IL-13 and CST1 were significantly higher ($p < 0.05$) in eosinophil^{high} versus eosinophil^{low} COPD patients (figure 1); fold change differences were 3.1, 2.0, 1.8 and 3.5 for the bronchoscopy cohort and 28.2, 9.4, 6.0 and 79.3 for the validation cohort respectively. POSTN and SERPINB2 expression were not different between groups (supplementary figure S1). ICS use and smoking status did not influence gene expression (data not shown). There was a significant positive correlation between a four-gene mean and sputum eosinophil counts in both cohorts (ρ 0.6 to 0.7 and $p < 0.001$; supplementary figure S1), but not other cell types (data not shown).

We report that eosinophilic COPD is associated with a profile of T2 inflammation in both bronchial epithelium and sputum samples; this was consistently observed in sputum samples from two independent cohorts. We tested six genes associated with T2 inflammation in asthma, and observed increased expression of four of these genes (CLCA1, CCL26, IL-13 and CST1) in eosinophil^{high} compared to eosinophil^{low} COPD patients. The strengths of this study include a validation cohort, and different lung samples (bronchial epithelium and sputum). The positive signals were > 1.8 fold higher (gene expression) in the eosinophil^{high} group. Different techniques (RNA sequencing and RT-qPCR) were used to analyse sputum samples, but the conclusions and relative fold changes showed the same pattern, adding confidence to our observations. The association between eosinophils and IL-13 expression levels suggests pathways linking eosinophilic inflammation to airway remodelling and mucus secretion (6), discussed further in the online supplement.

These results indicate a wider profile of T2 inflammation in eosinophilic COPD that extends to mechanisms including IL-13 driven pathways. While T2 inflammation is well recognised in

asthma, we now provide further evidence of T2 inflammation in a subset of COPD patients. The components of T2 inflammation described here may represent therapeutic targets.

REFERENCES

1. Kolsum U, Damera G, Pham TH, et al. Pulmonary inflammation in patients with chronic obstructive pulmonary disease with higher blood eosinophil counts. *J Allergy Clin Immunol*. 2017;140(4):1181-4 e7.
2. Singh D, Bafadhel M, Brightling CE, et al. Blood Eosinophil Counts in Clinical Trials for Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med*. 2020;202(5):660-71.
3. Christenson SA, Steiling K, van den Berge M, et al. Asthma-COPD overlap. Clinical relevance of genomic signatures of type 2 inflammation in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2015;191(7):758-66.
4. George L, Taylor AR, Esteve-Codina A, et al. Blood eosinophil count and airway epithelial transcriptome relationships in COPD versus asthma. *Allergy*. 2020;75(2):370-80.
5. Woodruff PG, Modrek B, Choy DF, et al. T-helper type 2-driven inflammation defines major subphenotypes of asthma. *Am J Respir Crit Care Med*. 2009;180(5):388-95.
6. Atherton HC, Jones G, Danahay H. IL-13-induced changes in the goblet cell density of human bronchial epithelial cell cultures: MAP kinase and phosphatidylinositol 3-kinase regulation. *Am J Physiol Lung Cell Mol Physiol*. 2003;285(3):L730-9.

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Conflicts of Interest

AH has received personal fees from Chiesi. AB, SW, NJ, GL, UK and TS have no conflicts of interest. T-HP, SS, CM and PN are employees of AstraZeneca. DS has received personal fees from AstraZeneca, Boehringer Ingelheim, Chiesi, Cipla, GlaxoSmithKline, Glenmark, Menarini, Mundipharma, Novartis, Peptinnovate, Pfizer, Pulmatrix, Therevance and Verona.

Table 1. Clinical Characteristics of the Bronchoscopy and Validation cohorts

Characteristic	Bronchoscopy Cohort			Validation Cohort		
	Eosinophil ^{low}	Eosinophil ^{high}	P value	Eosinophil ^{low}	Eosinophil ^{high}	P value
Number	17	20	N/A	18	15	N/A
Age (y)	62 ± 6	62 ± 4	0.7	64 ± 8	67 ± 9	0.3
Gender: Male (%)	65	70	0.7	44	73	0.1
BMI (kg/m ²)	27 ± 5	25 ± 4	0.2	25 ± 5	28 ± 4	0.06
Current smokers (%)	42	60	0.2	61	20	0.01
Pack-years history	43 ± 15	38 ± 14	0.3	48 ± 19	45 ± 25	0.7
ICS use (%)	82	55	0.08	39	93	0.02
ICS dose	0 (0-2000)	800 (0-2000)	0.009	1000 (0-2000)	600 (0-2000)	0.35
Post bronchodilator FEV ₁ (L)	1.9 ± 0.4	1.9 ± 0.4	0.9	1.7 ± 0.6	1.8 ± 0.5	0.6
Post bronchodilator FEV ₁ (% predicted)	64 ± 12	66 ± 11	0.6	67 ± 17	66 ± 16	0.8
Post bronchodilator FVC (L)	3.8 ± 1.3	3.6 ± 0.9	0.5	3.2 ± 1	3.3 ± 0.7	0.7
Post bronchodilator FEV ₁ /FVC ratio	50 ± 10	53 ± 7	0.3	53 ± 12	55 ± 11	0.7
Reversibility (mL)	207 ± 189	217 ± 154	0.9	166 ± 142	145 ± 147	0.7
FeNO ₅₀ (ppm)	16 ± 8.4	23 ± 15	0.2	9.2 ± 5.2	14.9 ± 8.1	0.02
Atopy (% positive)	0	0	N/A	6	13	0.4
Total SGRQ	42 ± 17	37 ± 21	0.4	56 ± 16	55 ± 16	0.9
mMRC	1.8 ± 1.0	1.3 ± 1.2	0.2	3.6 ± 0.7	3.7 ± 0.6	0.6
CAT	19 ± 9	17 ± 8	0.4	22 ± 7	22 ± 6	1
Exacerbation rate, 12 mo prior	0.8 (0-3)	0.7 (0-3)	0.6	0.8 (0-4)	1.6 (0-4)	0.04
Blood Eosinophils	10 ± 3	432 ± 144	<0.0001	9 ± 3	350 ± 240	<0.0001
Sputum Cell Counts						

Total (x10 ⁶ /g)	5.6(0.1-26.3)	5.9 (0.5-32.4)	0.9	1.9 (0.2-11.4)	2.4 (0.8-12.6)	0.2
Neutrophil (x10 ⁶ /g)	3.2(0.1-20.8)	3.6 (0.2-29.6)	0.9	3.9 (0.5-24.4)	5.4 (0.4-16.6)	0.8
Macrophage (x10 ⁶ /g)	1.3(0.01-4.6)	1.2 (0.2-7.7)	0.6	1.4 (0.3-3.5)	1.3 (0.4-4.5)	0.5
Eosinophil (x10 ⁶ /g)	0.02(0.0-0.2)	0.3 (0.01-4.2)	<0.001	0.04 (0.00 – 0.4)	0.3 (0.1-1.3)	<0.001
Lymphocyte (x10 ⁶ /g)	0.02(0.0-0.1)	0.02 (0.0-0.2)	0.4	0.04 (0.0-0.6)	0.01 (0.0-0.2)	0.4
Epithelial (x10 ⁶ /g)	0.04(0.0-0.7)	0.03 (0.0-1.0)	0.7	0.1 (0.02-0.6)	0.2 (0.04-1.6)	0.07
Eosinophils (%)	0.5 (0-2.5)	4.5 (0.3-70)	<0.0001	0.5 (0-2)	6.5 (2.8-16.5)	<0.0001
Neutrophils (%)	81 (17-91)	63 (15-91)	0.06	67 ± 20	65 ± 18	0.8
Macrophages (%)	17 (5-82)	23 (4-63)	0.6	30 ± 19	23 ± 15	0.3
Lymphocytes (%)	0.5 (0-2.3)	0.3 (0-2.5)	0.3	0.3 (0-2.8)	0.3 (0-3.5)	0.8
Epithelial (%)	1 (0-8)	1 (0-8)	.85	2 (0.3-10)	3 (0.3-17)	0.08

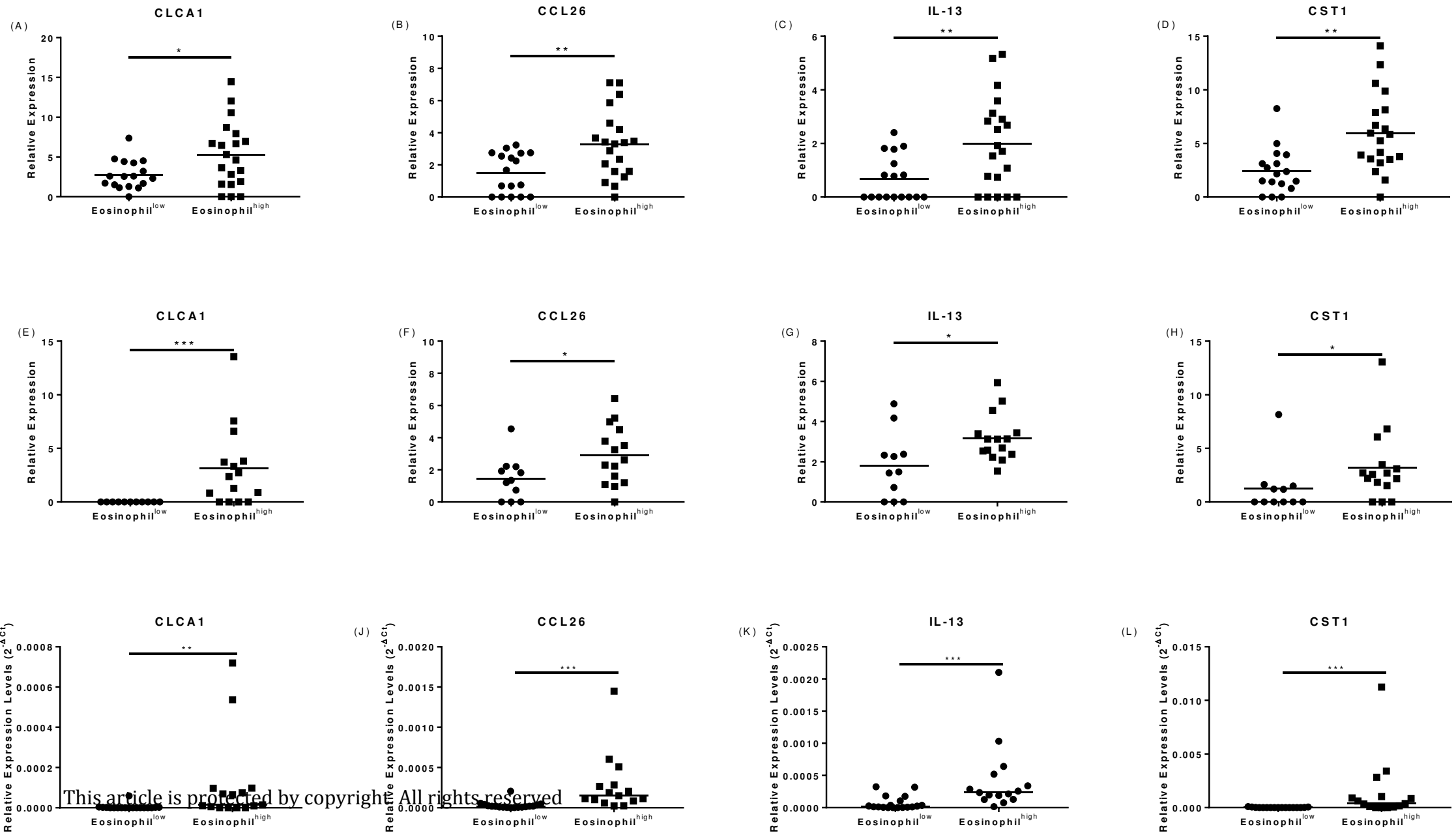
Data presented as %, mean ± standard deviation, or median (range).

BMI, body mass index; ICS, inhaled corticosteroids; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; FeNO₅₀, fractional exhaled nitric oxide at 50 mL⁻¹ sec flow rate; PPM, parts per million; SGRQ, St George's Respiratory Questionnaire; mMRC, modified Medical Research Council; CAT, COPD Assessment Test.

FIGURE LEGEND

Figure 1. T2 gene expression in bronchial brushings and sputum from the bronchoscopy and validation cohorts. RNA sequencing was used to examine the expression of CLCA1 (A, E, and I), CCL26 (B, F and J), IL-13 (C, G and K), CST1 (D, H and L), in RNA isolated from bronchial brushings (A - D) and sputum cells (E - H) from the bronchoscopy cohort and sputum cells from the validation cohort (I - L). Data presented as individual data points with mean (A - H) or median (I - L) where *, ** and *** = significant difference (p<0.05, p<0.01 and p<0.001 respectively).

For A –H gene expression was normalised to the transcript length for each gene. For I – L gene expression was normalised to GAPDH.



Online Supplement

Type 2 Inflammation in Eosinophilic Chronic Obstructive Pulmonary Disease

Methods

Study Subjects

COPD patients aged >40 with a smoking history of >10 pack-years, a post-bronchodilator forced expiratory volume in 1 sec (FEV₁) and forced vital capacity (FVC) ratio <0.7, and with no history of asthma were recruited. Patients receiving oral corticosteroids or antibiotics within six weeks of the study were excluded. Atopy was determined by a positive skin prick test against house dust mite extract, cat dander or grass pollen.

Two cohorts were involved in this analysis: a bronchoscopy and validation cohort. The bronchoscopy cohort was previously reported (n=20 eosinophil^{high} and n=21 eosinophil^{low}); here we include samples from n=20 eosinophil^{high} and n=17 eosinophil^{low} COPD patients with sufficient RNA extracted for analysis from either bronchial brushing and / or sputum samples. The refined definition of eosinophil^{high} and eosinophil^{low} in the validation cohort aimed to enrich the population for patients with concordant low or high eosinophils in the blood and airways. In the validation cohort, patients were required to have MRC scores ≥ 2 and CAT >15, as part of a larger study of highly symptomatic COPD patients.

Sputum induction and bronchoscopy sampling of bronchial brushings were conducted as previously reported (1). Venepuncture was conducted on the same visit as sputum collection. Sample collection was approved by the local research ethics committees (REC): bronchoscopy cohort; South Manchester REC 06/Q1403/156 (brushings), Tameside and Glossop local REC 05/Q1402/41 (sputum) and NRES Committee North West – Preston 10/H1016/25 (blood). Validation cohort; NRES Committee North West – Preston 16/NW/0836. All subjects provided written informed consent.

Sputum Processing

Sputum was induced using 3%, 4% and 5% saline, inhaled in sequence for 5 min, up to 15 min via an ultrasonic nebuliser (EASyneb II, Flaemnouva, Italy) and processed as previously reported (1).

Statistical Analysis

Statistical analyses were performed using GraphPad InStat software (GraphPad Software Inc., La Jolla, California, USA). Data distributions were determined by the D'Agostino and Pearson normality test. Comparisons between groups were made by an unpaired t-test (parametric data; bronchoscopy cohort), Mann-Whitney test (non-parametric data; validation cohort) or Chi-squared test where indicated. T2 genes were summarized per-patient by taking the mean of expression of the four genes (CLCA1, CCL26, IL-13 and CST1) per patient (four gene signature). Pearson or Spearman correlations were performed to determine associations between T2 signature means and sputum cell percentages and sputum cell counts. **RNA Extraction and cDNA Analysis**

Bronchoscopy Cohort

Bronchial brushings were collected in bronchial epithelial basal medium (Lonza, Slough, UK) and stored on ice before centrifugation (400 g for 10 min at 4°C). The cell pellet was re-suspended in RLT buffer (Qiagen) with β -mercaptoethanol added according to manufacturer's instructions. Following removal of the supernatants during sputum processing, the remaining sputum cell pellet was re-suspended in RLT buffer plus β -mercaptoethanol. Total RNA was extracted from bronchial epithelial brushings and sputum cells using ZR RNA MicroPrep kit (Zymo Research, Orange, CA, USA) and RNA-seq libraries were prepared using TruSeq Stranded mRNA Prep kit (Illumina, San Diego, CA, USA) per manufacturers' protocols. To confirm RNA quality, RIN scores were evaluated using the 2100 Bioanalyzer system (Agilent, Santa Clara, CA, USA). Paired-end sequencing (75 base pairs per end) with sequencing depth at 80 million reads was performed on the HiSeq2000 platform (Illumina,

San Diego, CA, USA) to generate FASTQ files. These were aligned to human genome (version HG19) using HiSAT2 (John Hopkin's University, Baltimore, USA) and SAMtools (Genome Research Limited, Cambridge, UK). Normalized read counts were generated per transcript using DESeq2 (2). Read counts were then transformed to log2 scale (after adding 1 to account for zero read counts).

Validation Cohort

Following removal of the supernatants, the sputum cell pellet was re-suspended in RLT buffer plus β -mercaptoethanol. Total RNA was purified from cell lysates using RNeasy kits (Qiagen, Crawley, UK) according to manufacturer's instructions. DNA contamination was prevented by on-column addition of DNase (Qiagen, Crawley, UK) according to manufacturer's instructions. TaqMan reverse transcription-PCR (RT-PCR) was performed on 50 ng of RNA using the Verso 2-step RT-qPCR kit (Thermo Scientific) according to manufacturer's instructions. The resulting cDNA was reacted with Absolute blue qPCR mix (Thermo Scientific) according to manufacturer's instructions in 25 μ l reactions containing pre-made ABI Taqman gene expression assays for POSTN (Hs01566750_m1), CLCA1 (Hs00976287_m1), SERPINB2 (Hs01010736_m1), CCL26 (Hs00171146_m1), IL-13 (Hs00174379_m), CST1 (Hs0060691_m1) and the endogenous control was glyceraldehyde-3phosphate dehydrogenase (GAPDH) (Catalogue no: 4352934E) (Applied Biosystems, Warrington, UK). Controls without RT-enzyme showed there was no genomic DNA contamination. Thermal cycling was carried out on a Stratagene MX3005P (Agilent Technologies, West Lothian, UK). Relative expression levels were determined using the $2^{-\Delta C_t}$ (cycle threshold of gene of interest minus cycle threshold of GAPDH). Zero values represent undetectable gene expression.

Additional Discussion

George et al, using an unbiased approach, showed an association between bronchial epithelial T2 gene expression and blood eosinophil counts in COPD patients (3). There was limited overlap between the genes associated with blood eosinophil counts in asthma and COPD patients. We selected a panel of well accepted T2 associated genes in asthma (4-6). Our hypothesis-driven approach confirms that T2 inflammation in eosinophilic COPD is not identical to T2 inflammation in asthma, as only 4 out of 6 genes were differentially regulated in COPD. Gene expression studies enable researchers to maximise sample usage and have proven important to identify T2 asthma sub-populations. However, future studies should confirm our observations at the protein level as gene expression may not always accurately reflect protein expression in the airways. Unbiased bronchial epithelial gene expression studies have shown that both CCL26 and CST1 expression are associated with blood eosinophil counts in COPD patients (3, 7). Our results confirm these observations, and show differential regulation of IL-13 and CLCA1. The four-gene T2 signature (CLCA1, CCL26, IL-13 and CST1) was positively correlated with sputum eosinophil numbers in both cohorts, with the same relative fold change pattern in both cohorts suggesting a hierarchy of T2 gene expression. These findings should be confirmed in larger cohorts, or using other analytical methodology such as gene set variation analysis.

We investigated the expression of the selected genes in whole sputum and bronchial epithelial samples. However, due to the methodology used, we were unable to discern which specific cell types are driving these observations. Future studies should look to examine which sputum cell types or specific bronchial epithelial cells (eg secretory cells, basal cells, ciliated cells) express these genes.

IL-13 and CLCA1 induce goblet cell hyperplasia and mucus hypersecretion (8). Previous studies have implicated IL-13 in the development of COPD pathophysiology including mucus hypersecretion (9), airway remodelling and possibly emphysema development (10). Our results indicate that any IL-13 driven pathophysiology will be greater in eosinophilic COPD patients.

POSTN and SERPINB2 are IL-13 inducible genes. We observed upregulation of IL-13 expression in eosinophilic COPD, but no statistical differences in POSTN and SERPINB2 expression; numerical differences were observed in bronchial epithelial gene expression ($p=0.06$ and $p=0.07$ respectively) and larger study groups may have yielded significant differences. Nevertheless, current smoking may repress the expression of these genes (7), while epithelial gene expression of POSTN and SERPINB2 do not associate with blood eosinophils in COPD patients (3). While we did not observe any effect of

current smoking on these genes, the overall evidence indicates that POSTN and SERPINB2 do not form part of a reliable T2 signature in COPD. This highlights the differences in T2 inflammation between asthma and eosinophilic COPD, which probably arise due to the different inflammatory milieu in COPD patients e.g. bacteria, oxidative stress and smoking.

CCL26 induces eosinophil chemotaxis, but may also have a protective role in COPD by exerting anti-bacterial activity against pathogens including non-typeable *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* (11). This may be relevant to the inverse relationship between pulmonary eosinophil counts and the levels of pathogenic bacteria in the airways of COPD patients (12). CST1 may also help protect the lungs from pathogenic colonisation by protecting tight junction integrity in epithelial cells (13). CST1 can also induce IL-5 and CCL11 (eotaxin-1) release from stromal cells (14), thus further perpetuating eosinophilic inflammation.

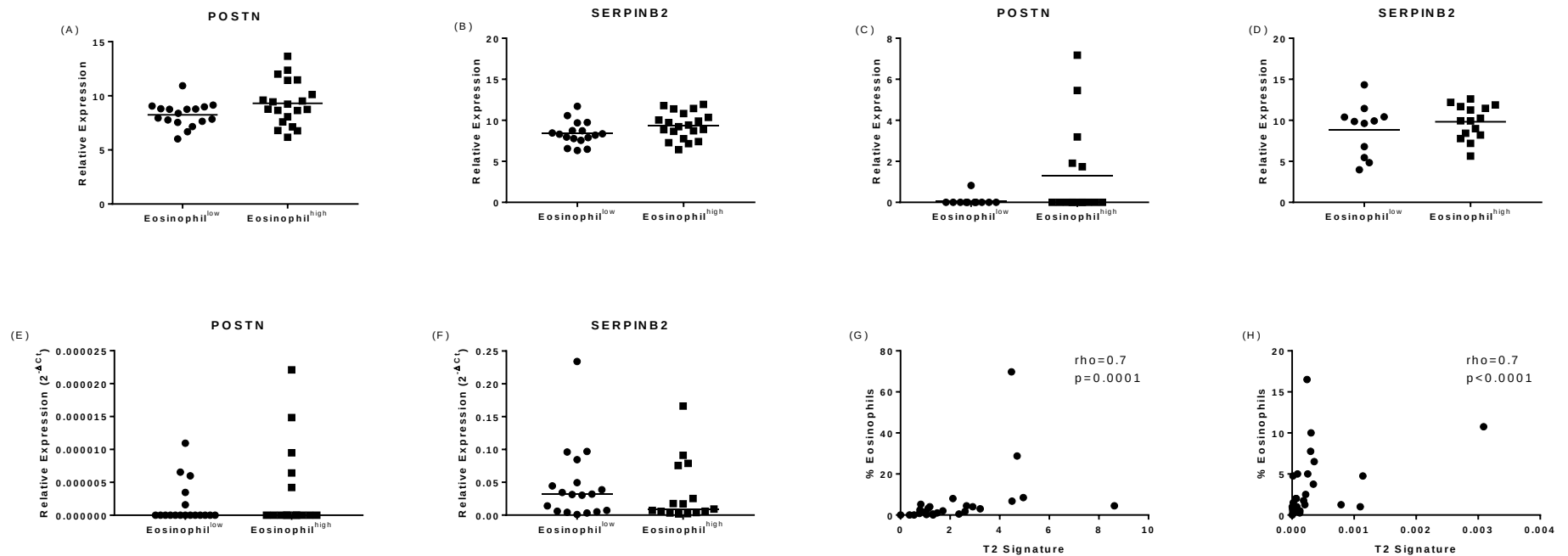
Slightly different definitions were used for eosinophil^{high} versus eosinophil^{low}, as the validation cohort also had a sputum eosinophil criterion. Our previous publication showed that most, but not all, COPD patients in the blood eosinophil^{high} group had raised lung eosinophils; 16/20 patients in the bronchoscopy cohort with >250 eosinophils μL^{-1} had >3% sputum eosinophils whereas no patients with <150 eosinophils μL^{-1} had >3% sputum eosinophils. We therefore further enriched the specificity of our selection of eosinophil^{high} and eosinophil^{low} individuals by adding a sputum cell criterion in the validation cohort. The cohorts also had different inclusion criteria regarding symptoms, leading to a more symptomatic group in the validation cohort. There was no difference in FEV₁ between cohorts, which may not be surprising as symptoms and FEV₁ are poorly correlated in COPD (15).

Randomised clinical trials in COPD patients with a history of exacerbations have consistently shown higher blood eosinophil counts are associated with increased exacerbation rates in patients randomised to receive no ICS treatment (16). This relationship has been less consistent in observational cohort studies due to the confounding effects of ICS use and different exacerbation histories (17). Our bronchoscopy cohort showed no difference in exacerbation rates between groups. However, in the validation cohort there was a higher exacerbation rate and greater ICS use in the eosinophil^{high} group. The limited sample size cautions against over-interpretation of these findings. Clinical trials of monoclonal antibodies directed against IL-5 or the IL-5 receptor in COPD patients with frequent exacerbations have shown variable results on exacerbation prevention, with both positive and negative results reported, although efficacy appears to increase with higher baseline eosinophil counts (18, 19). We speculate our results provide potential mechanistic insights into these findings; eosinophil^{high} COPD patients exhibit a profile of inflammation beyond simply raised eosinophil numbers, therefore, targeting eosinophils alone without further refinement of the COPD population may have limited therapeutic benefit. ICS have a relatively large effect size on exacerbation rate reduction in patients with blood eosinophil levels > 300 μL^{-1} , which may be due to broad effects on T2 inflammation. However, recent analysis of the Phase 3 COPD clinical trials with the anti-IL-5R α monoclonal antibody, benralizumab, identified a COPD sub-group with a history of frequent exacerbations while receiving triple therapy in addition to high blood eosinophils that may achieve greater benefit from this targeted treatment (20). These may represent a subgroup of T2 high patients who have poor response to ICS, similar to T2 high asthma patients who remain symptomatic despite high ICS doses. The strong correlation between eosinophil numbers and the four gene mean suggests a scaling in intensity, possibly explained by a variation of T2 inflammation over time, similar to blood and lung eosinophil numbers (21). Identifying which factors drive T2 inflammation in these patients may facilitate targeted therapies.

We observed some differences in clinical characteristics between the bronchoscopy and validation cohorts including ICS use, mMRC score and the number of exacerbations in the previous 12 months. Despite these differences, specific gene expression changes including the order of magnitude of fold changes were consistent, giving confidence that eosinophilic inflammation is strongly associated with T2 inflammation in COPD patients.

References

1. Higham A, Karur P, Jackson N, et al. Differential anti-inflammatory effects of budesonide and a p38 MAPK inhibitor AZD7624 on COPD pulmonary cells. *Int J Chron Obstruct Pulmon Dis*. 2018;13:1279-88.
2. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15(12):550.
3. George L, Taylor AR, Esteve-Codina A, et al. Blood eosinophil count and airway epithelial transcriptome relationships in COPD versus asthma. *Allergy*. 2020;75(2):370-80.
4. Silkoff PE, Laviolette M, Singh D, et al. Identification of airway mucosal type 2 inflammation by using clinical biomarkers in asthmatic patients. *J Allergy Clin Immunol*. 2017;140(3):710-9.
5. Woodruff PG, Boushey HA, Dolganov GM, et al. Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids. *Proc Natl Acad Sci U S A*. 2007;104(40):15858-63.
6. Woodruff PG, Modrek B, Choy DF, et al. T-helper type 2-driven inflammation defines major subphenotypes of asthma. *Am J Respir Crit Care Med*. 2009;180(5):388-95.
7. Christenson SA, Steiling K, van den Berge M, et al. Asthma-COPD overlap. Clinical relevance of genomic signatures of type 2 inflammation in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2015;191(7):758-66.
8. Atherton HC, Jones G, Danahay H. IL-13-induced changes in the goblet cell density of human bronchial epithelial cell cultures: MAP kinase and phosphatidylinositol 3-kinase regulation. *Am J Physiol Lung Cell Mol Physiol*. 2003;285(3):L730-9.
9. Miotto D, Ruggieri MP, Boschetto P, et al. Interleukin-13 and -4 expression in the central airways of smokers with chronic bronchitis. *Eur Respir J*. 2003;22(4):602-8.
10. Doyle AD, Mukherjee M, LeSuer WE, et al. Eosinophil-derived IL-13 promotes emphysema. *Eur Respir J*. 2019;53(5).
11. Gela A, Kasetty G, Jovic S, et al. Eotaxin-3 (CCL26) exerts innate host defense activities that are modulated by mast cell proteases. *Allergy*. 2015;70(2):161-70.
12. Kolsum U, Donaldson GC, Singh R, et al. Blood and sputum eosinophils in COPD; relationship with bacterial load. *Respir Res*. 2017;18(1):88.
13. Fukuoka A, Matsushita K, Morikawa T, et al. Human cystatin SN is an endogenous protease inhibitor that prevents allergic rhinitis. *J Allergy Clin Immunol*. 2019;143(3):1153-62 e12.
14. Yan B, Lou H, Wang Y, et al. Epithelium-derived cystatin SN enhances eosinophil activation and infiltration through IL-5 in patients with chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol*. 2019;144(2):455-69.
15. Agusti A, Calverley PM, Celli B, et al. Characterisation of COPD heterogeneity in the ECLIPSE cohort. *Respir Res*. 2010;11:122.
16. Bafadhel M, Peterson S, De Blas MA, et al. Predictors of exacerbation risk and response to budesonide in patients with chronic obstructive pulmonary disease: a post-hoc analysis of three randomised trials. *Lancet Respir Med*. 2018;6(2):117-26.
17. Singh D, Agusti A, Anzueto A, et al. Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Lung Disease: the GOLD science committee report 2019. *Eur Respir J*. 2019;53(5).
18. Criner GJ, Celli BR, Brightling CE, et al. Benralizumab for the Prevention of COPD Exacerbations. *N Engl J Med*. 2019;381(11):1023-34.
19. Pavord ID, Chanez P, Criner GJ, et al. Mepolizumab for Eosinophilic Chronic Obstructive Pulmonary Disease. *N Engl J Med*. 2017;377(17):1613-29.
20. Criner GJ, Celli BR, Singh D, et al. Predicting response to benralizumab in chronic obstructive pulmonary disease: analyses of GALATHEA and TERRANOVA studies. *Lancet Respir Med*. 2020;8(2):158-70.
21. Higham A, Leow-Dyke S, Jackson N, Singh D. Stability of Eosinophilic Inflammation in COPD Bronchial Biopsies. *Eur Respir J*. 2020.



Supplementary figure 1. T2 gene expression in bronchial brushings and sputum from the bronchoscopy and validation cohorts. RNA sequencing was used to examine the expression of POSTN (A, C and E) and SERPINB2 (B, D and F), in RNA isolated from bronchial brushings (A and B) and sputum cells (C and D) from the bronchoscopy cohort and sputum cells from the validation cohort (E and F). Data presented as individual data points with mean (A-D) or median (E-F). The percentage of sputum eosinophils was correlated with the T2 four gene signature (CLCA1, CCL26, IL-13 and CST1) in the bronchoscopy cohort (G) and validation cohort (H)