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Article type : Letter to the Editor

Inhibition of CpG methylation improves the barrier integrity of bronchial epithelial cells in asthma.

To the Editor:

Asthma is a complex and heterogeneous chronic airway inflammatory disease with the involvement of environmental factors through epigenetic mechanisms.¹ Accordingly, repeated injury, repair and regeneration of the airway epithelium following exposure to environmental factors and inflammation results in histological changes and functional abnormalities in the airway mucosal epithelium, which are associated with the pathophysiology of asthma.² Epigenetics is defined by heritable changes in gene expression without changes in the DNA sequence.³ Regulation of gene expression is mediated by different mechanisms such as DNA methylation, histone modifications and RNA-associated silencing by small non-coding RNAs. CpG sites are dinucleotides consisting of guanine and cytosine concentrated in clusters referred to CpG islands found at important regulatory sites, such as promoter and enhancer regions.⁴ Their *de novo* methylation occurs in response to various cellular stressors and signals by DNA methyltransferases (DNMT3a and 3b), which add a methyl group to position 5 of cytosine residues at the CpG site. During DNA replication both of the separated strands of DNA carry one methylated cytosine to be used as a template for duplication. Daughter DNA duplex strands will thus be hemi-methylated, which is recognized by a different DNA methyltransferase isoform (DNMT1). Because DNA methylation is a reversible process, the DNMTs are considered as a therapeutic target. Several DNMT inhibitors have been

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/ALL.14667](https://doi.org/10.1111/ALL.14667)

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identified recently, among the non-nucleoside inhibitors, 4-aminoquoline-based inhibitors, such as SGI-1027 showed potent inhibitory activity. SGI-1027 occupies the binding site of DNMTs resulting in the prevention of access of target DNA to the substrate binding pocket.⁵

We have demonstrated in previous studies from our laboratory that human primary bronchial epithelial cells (HBEC) isolated from patients with asthma showed lower barrier integrity compared to controls.⁶ Seeing that important role of DNA methylation in lung cells and asthma pathogenesis by other groups⁷ we investigate the level of global methylation in HBEC of control and asthma samples for the long interspersed nuclear element-1 (LINE-1) methylation levels (Figure 1A). HBEC from asthma patients showed a tendency for higher global methylation levels, together with higher expression of 5-methylcytosine (5-mc) in immunofluorescence staining (Figure 1B). Next, we performed methylation profiling (Illumina Infinium EPIC array) to investigate genes methylated in ALI cultures of HBEC. Interestingly, in a highly methylated group of top 100 genes, we found many genes associated with cell growth, ion transport, and cytoskeletal remodeling (Figure S1). Several pro-inflammatory cytokines had lower methylation levels, whereas regulatory cytokines, such as *IL10RA* and *TGFBR2* were methylated (Figure S2). In genes involved in the epigenetic process, we found members of histone demethyltransferases, histone deacetylases in both groups, but interestingly ten-eleven translocation enzyme (*TET1*), which can reverse CpG methylation was methylated. On the other side, genes belong to protein arginine methyltransferases (PRMT) were not methylated. Many genes specifically involved in the regulation of bronchial barrier integrity showed higher methylation (e.g., TJ family members: *AMOTL1*, *CLDN11*, *CLDN18*, *MAGI1*, *TJP2*, *JAM3*, actin protein: *ACTB*, a component of the cytoskeleton: *TUBA1C*, *ROCK2*, *LLGL1*. We kept our attention on the methylated epigenetic and tight junction (TJ) genes and further focused on TJs, especially zonula occludens and claudins which showed higher methylation in contrast to occludin, which was not methylated (Figure 1C). As higher methylation levels were observed in HBEC of asthmatic origin, we inhibited the DNA methyltransferase enzyme with a specific inhibitor, SGI-1027, to demonstrate the role of CpG methylation on epithelial barrier integrity. ALI cultures were treated with the DNA methyltransferase inhibitor for 72 hours. Significantly decreased

expression of 5-mc was observed after 48 hours of DNA-methyltransferase inhibition, demonstrating that the methylation of 5-methylcytosine (5-mc) in bronchial epithelium was reversed (Figure 2A). This prompted us to investigate the changes triggered by the inhibitor in epithelial cells. Further experiments showed increased transepithelial electric resistance (TER) in bronchial epithelial cells, in ALI from asthmatic donors after 48 hours of DNMT inhibition (Figure 2B, Figure S5A). The link between barrier integrity and TER results were confirmed by the significantly decreased paracellular passage of FITC-labelled 4kD dextran after inhibition of DNMTs (Figure 2C, Figure S5B). The reconstitution of TER in asthmatic ALI was associated with decreased protein DNMT1 expression and increased ZO-1 and claudin-18 proteins (Figure 2D). We also observed increased claudin-4, but not occludin expression upon DNMT inhibition (Figure S3). Increased expression of ZO-1 with an intact and honeycomb-like structure in the immunofluorescence staining of bronchial epithelial cells confirmed the effect on protein expression of bronchial epithelial barrier in asthma donors (Figure S4).

Defective epithelial barrier has been established in asthma in addition to several chronic inflammatory diseases.⁸ Direct targeting of the epithelial barrier leakiness for the treatments represents an important target, however so far there is no treatment possibility targeting epigenetic mechanisms. The present study demonstrates an increased global methylation level in HBEC from asthmatic individuals. CpG methylation of specific genes is essential for the defect of epithelial barrier integrity, which is reversed upon DNMT inhibition. The inversion of CpG methylation, restores leakiness in the epithelium in asthma by increasing TER, decreasing paracellular flux and improves the structure of bronchial epithelial cells by increasing the expression of TJ proteins. The better understanding of the importance of epigenetic memory in chronic tissue inflammatory diseases together with the availability of treatment modalities targeting epigenetic mechanisms and transition of these molecules into the clinical studies may lead to curative treatment of allergic and autoimmune inflammatory diseases.⁹

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Conflict of interest:

The authors declare that they have no conflicts of interest.

Founding sources:

Supported by Swiss National Science Foundation grants 310030_156823, and 320030_176190.

Word count: 901

Keywords: asthma, tight junction, CpG methylation, DNA methyltransferases,

Abbreviations:

ALI: air-liquid interface,

BEGM: bronchial epithelial growth medium,

DNMT: DNA methyltransferase

FITC: fluorescein isothiocyanate,

HBEC: human bronchial epithelial cells,

LINE-1: long interspersed nuclear element-1

5-mc: 5-methylcytosine

TER: transepithelial electric resistance,

ZO: zonula occludens

FIGURE LEGENDS:

Figure 1. Increased global methylation in bronchial epithelial cells in asthma

(A) Global LINE-1 methylation levels in bronchial epithelial cells from healthy control (n=4) and asthma patients (n=3) on the baseline. (B) Representative

immunofluorescence staining and data showing the mean intensity of ALI cultures of differentiated bronchial epithelial cells from controls (n=5) and asthma patients (n=8) for 5-methylcytosine (5mc). Data are presented as mean±SD, (**P < 0.01), Mann Whitney U test. (C) Heatmaps of genes belong to the epigenetic group, and genes involved in the regulation of epithelial integrity, control n=4, asthmatic n=3.

Figure 2. DNA methyltransferase inhibitor decreases global methylation and improve the barrier integrity of bronchial epithelial cells in asthma

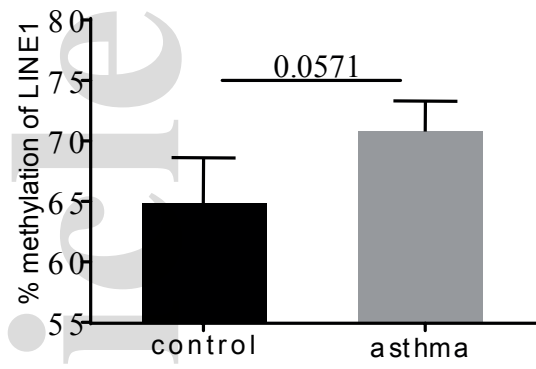
Representative immunofluorescence staining of 5-methylcytosine (5mc) and data showing the mean intensity in asthmatic ALI cultures of HBEC treated with 8 µM DNA methyltransferase inhibitor, SGI-1027 for 48 hours, (n=8) (A). (B) Increased TER in ALI cultures of HBEC from asthmatic individuals treated with 8 µM of DNA methyltransferase inhibitor-SGI-1027, n=5 per group. (C) The decreased paracellular flux of 4kDa FITC-dextran in response to 48 hours treatment of 8 µM DNA methyltransferase inhibitor SGI-1027, n=5 per group. (D) Western blots of DNMT1, ZO-1 and claudin-18 from ALI cultures of asthmatic HBEC treated with DNA methyltransferase inhibitor-SGI-1027 in different doses (2 µM, 4 µM, 8 µM, 16 µM), n=5. Data represent mean±SD, (*P < 0.05), (**P < 0.01), Mann Whitney U test.

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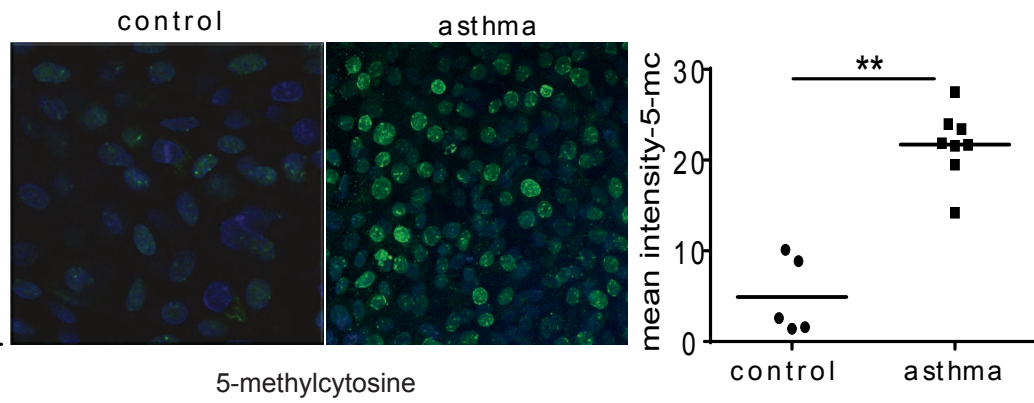
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A



B

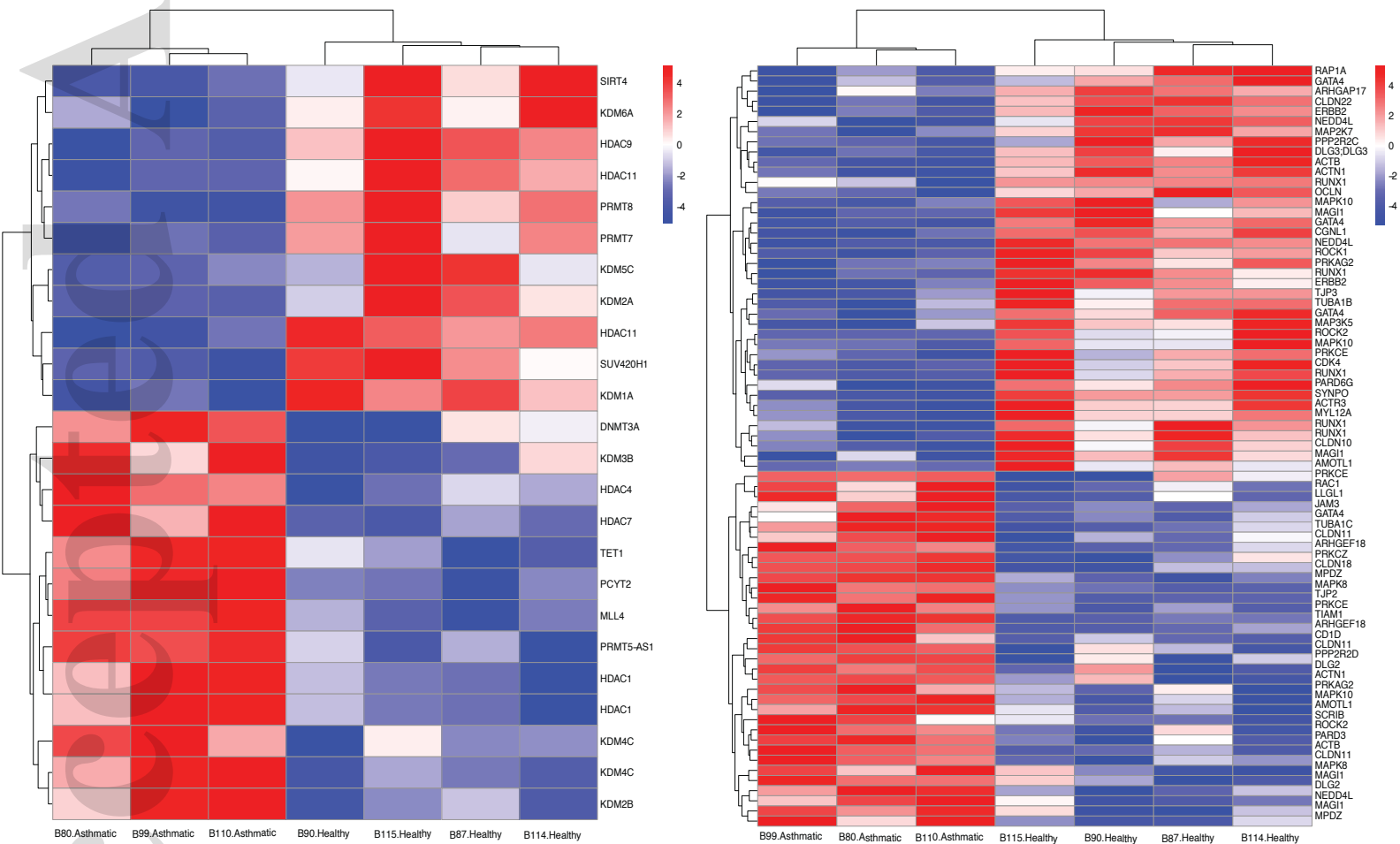
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C

Epigenetic

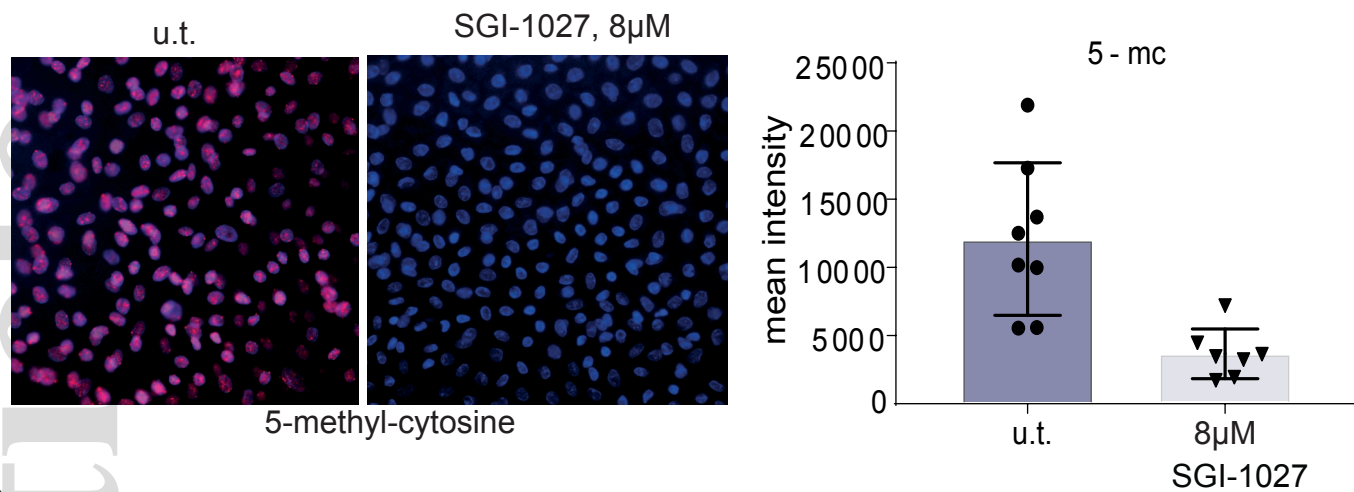
Epithelial barrier



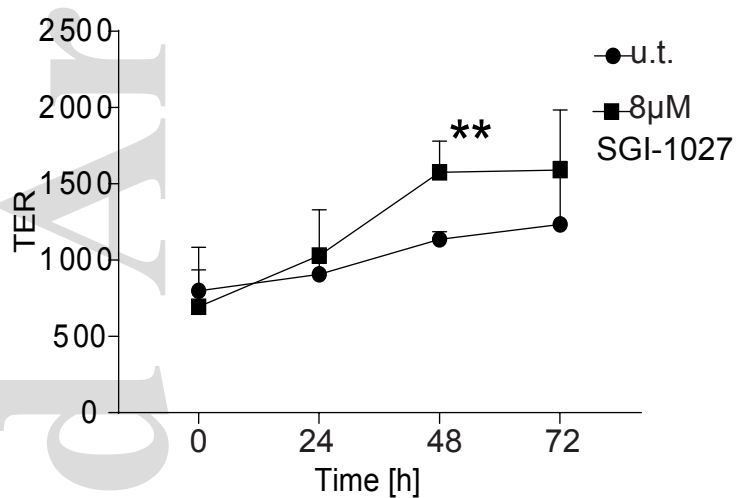
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Figure 1. Wawrzyniak et al.

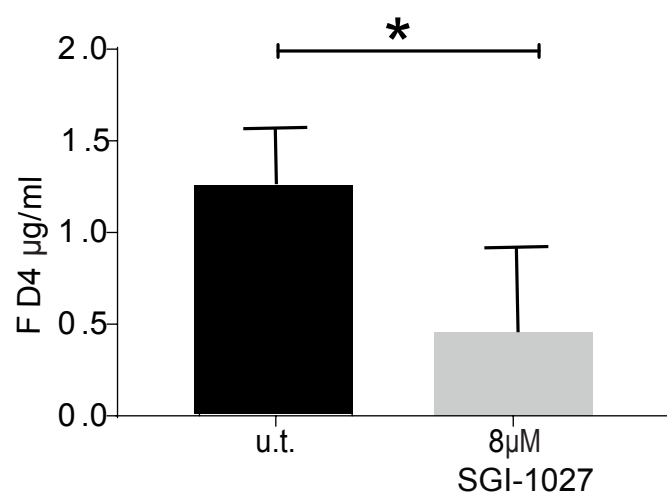
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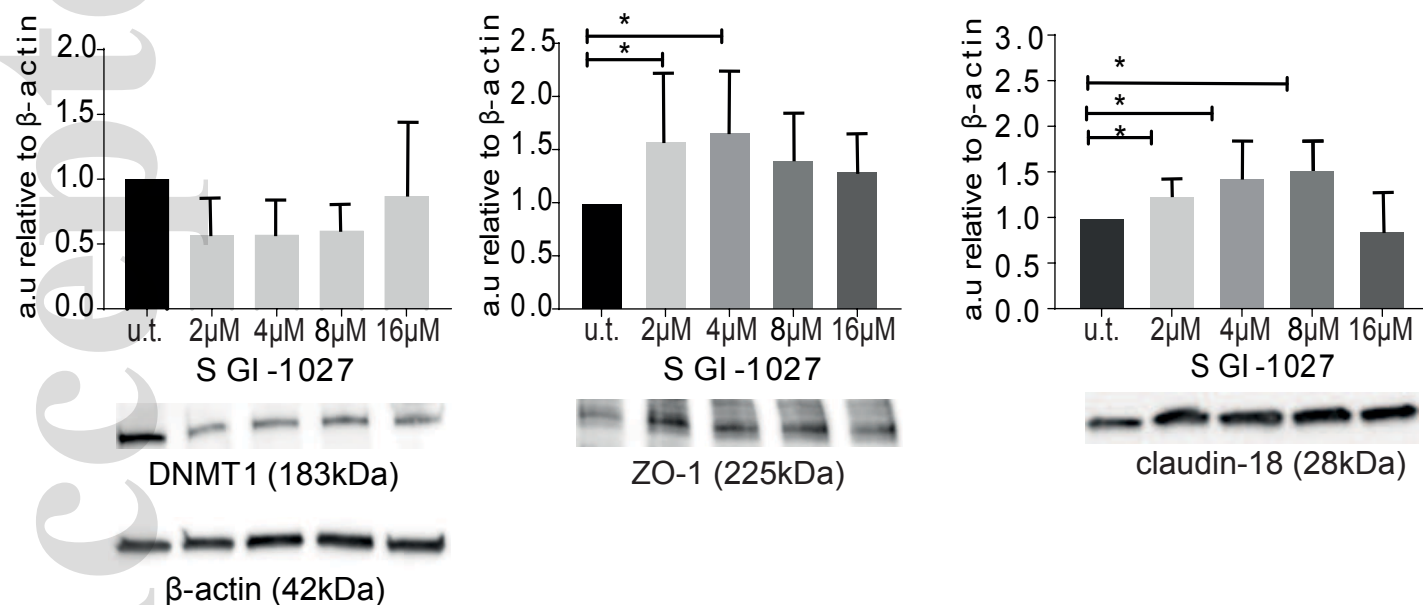


Figure. 2 Wawrzyniak et al.

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.

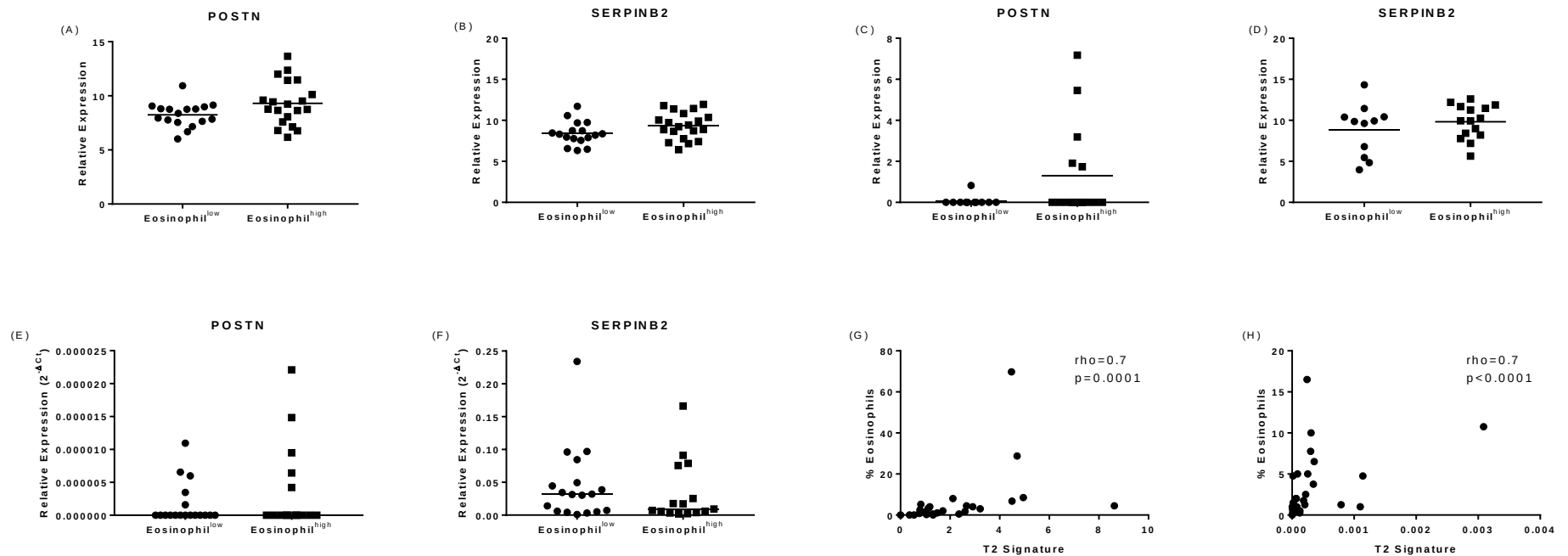
Slightly different definitions were used for eosinophil^{high} versus eosinophil^{low}, as the validation cohort also had a sputum eosinophil criterion. Our previous publication showed that most, but not all, COPD patients in the blood eosinophil^{high} group had raised lung eosinophils; 16/20 patients 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.

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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)