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**Contribution of airway eosinophils in airway wall remodeling in asthma:
role of *MMP-10* and *MET***

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Running title: Remodeling pathways in eosinophilic asthma

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Data repository

The transcriptomic data have been deposited in the Gene Expression Omnibus database, <http://www.ncbi.nlm.nih.gov/geo> (accession no. GSE76225 for gene expression data of bronchial biopsies).

Supplementary files

Supplementary figures (4) & tables (6)

Supplementary files of Acknowledgement

Abstract

Background: Eosinophils play an important role in the pathophysiology of asthma being implicated in airway epithelial damage and airway wall remodeling. We determined the genes associated with airway remodeling and eosinophilic inflammation in patients with asthma.

Methods: We analyzed the transcriptomic data from bronchial biopsies of 81 moderate-to-severe asthma patients of the U-BIOPRED cohort. Expression profiling was performed using Affymetrix arrays on total RNA. Transcription binding site analysis used the PRIMA algorithm. Localisation of proteins was by immunohistochemistry.

Results: Using stringent false discovery rate analysis, MMP-10 and MET were significantly overexpressed in biopsies with high mucosal eosinophils (HE) compared to low mucosal (LE) numbers. Immunohistochemical analysis confirmed increased expression of MMP-10 and MET in bronchial epithelial cells and in subepithelial inflammatory and resident cells in asthmatic biopsies. Using less-stringent conditions (raw p-value <0.05 , log2Fold Change >0.5), we defined a 73-gene set characteristic of the HE compared to the LE group. 33/73 genes drove the pathway annotation that included extracellular matrix (ECM) organization, mast cell activation, CC-chemokine receptor binding, circulating immunoglobulin complex, serine protease inhibitors and microtubule bundle formation pathways. Genes including MET and MMP10 involved in ECM organisation correlated positively with submucosal thickness. Transcription factor binding site analysis identified two

transcription factors, ETS-1 and SOX family proteins that showed positive correlation with MMP10 and MET expression.

Conclusion: Pathways of airway remodeling and cellular inflammation are associated with submucosal eosinophilia. MET and MMP-10 likely play an important role in these processes.

Key words:

Asthma, eosinophil, MMP10, MET, mast cell.

Messages: GRAPHIC VISUAL:

- Analysis of differentially-expressed genes in eosinophil-high biopsies of asthma yielded MMP10 and MET as potential drivers of airway wall remodeling.
- Important pathways include extracellular matrix organization, mast cell activation, C-C receptor binding and regulation of leukocyte activation.
- Bronchial eosinophils are important drivers of airway wall remodeling in asthma.

MMP10: Matrix metalloprotease 10 gene

MET: Hepatocyte growth factor receptor gene

Introduction

Asthma is a complex disease that is heterogeneous in its clinical presentation. In addition, its inflammatory component may vary between asthmatic subjects with the recognition of eosinophilic inflammation as being a specific feature of a particular type of asthma associated with a late onset non-atopic asthma, presence of nasal polyps, chronic airflow obstruction and likely to be treated with oral corticosteroid therapy(1). Asthmatic patients with eosinophilia as evidenced by an increase in blood or sputum eosinophil count are also at an increased risk of exacerbations and poorly-controlled asthma(2), and are also more likely to respond to corticosteroid therapy(3) and to specific anti-eosinophilic therapies such as anti-IL5 immunomodulators (4).

These observations indicate that eosinophils may play an important role in the pathophysiology of asthma. Eosinophils have been implicated in damaging the airway epithelium through the release of reactive oxygen species, eosinophil cationic protein and eosinophil peroxidase and have also been implicated in airway wall remodeling changes such as the increase in reticular subbasement membrane by direct interaction of eosinophils with fibroblasts(5, 6). In addition, IL-5 activation of eosinophils may lead to the production of cationic proteins that activate TGF β , MMP and PDGF release from bronchial epithelial cells(7). Indeed, anti-IL5 antibody therapy in asthmatics can lead to a reduction in extracellular matrix proteins in the subbasement membrane(8).

We hypothesized that eosinophils contribute to airway wall remodeling and inflammation in asthma. To this end, we analysed the transcriptome of bronchial biopsies of patients with asthma in relation to the degree of eosinophilic inflammation using data collected in the European U-BIOPRED cohort(9). We determined the differentially-expressed genes (DEGs) in the bronchial mucosa from patients with high levels of eosinophils compared to low levels of eosinophil density in their bronchial mucosa. We confirmed dysregulation of MMP10 and MET protein expression using immunohistochemistry and identified the transcription factors that control the expression of their genes.

Materials and methods

Clinical data of patients with bronchial biopsies suitable for immunohistochemistry

In this study, of 158 participants who underwent bronchoscopic procedure, 137 and 136 biopsies were suitable for immunohistochemistry and transcriptomic microarray analysis, respectively; where 123 were qualified for both, of whom 81 participants were moderate-to-severe asthma and 41 healthy controls (**Table S1**) from 11 European centres of the U-BIOPRED study (9). Expression profiling of the bronchial biopsies was performed using Affymetrix U133 Plus 2.0 (Affymetrix, Santa Clara, CA, USA) microarrays.

Pre-bronchodilator spirometry, exhaled nitric oxide (FeNO), sputum differential cell count, allergic status assessed by either skin prick tests or by RAST test to specific aeroallergens, serum total IgE, and differential blood count were measured(9). The study was

approved by the Ethics Committees of each recruiting centre and all participants gave written informed consent.

Immunohistochemistry of U-BIOPRED bronchial biopsies

Samples of bronchial biopsies from paraformaldehyde-preserved sections (6µm) were stained with monoclonal antibodies against CD3, CD4, CD8, neutrophil elastase and EG2. Cell counts were performed in a blinded fashion and expressed as number of positive cells/mm². These results have already been reported(10).

Immunohistochemical analysis of MET and MMP-10 in bronchial biopsies

In order to confirm the presence of MET and MMP10 in the airways of patients with asthma, bronchial biopsies kept at the National Heart & Lung Institute Biobank were obtained from 6 normal and 10 asthma (5 mild and 5 severe) subjects. Paraffin sections were immunostained with primary rabbit anti-MET antibody (1:200 dilution) (Abcam, Cambridge, UK, ab51067) and rabbit anti- MMP10 (1:100 dilution) (Abcam, ab189210) antibodies, and MET- and MMP10-positive cells were detected using peroxidase staining method. Non-specific rabbit IgG, polyclonal-isotype control (Abcam, ab171870) was used as a control primary antibody.

The immunostaining intensity for MET and MMP10 on bronchial epithelium was semi-quantitatively scored from 0-3 (0-negative, 1-weak, 2-moderate and 3-strong staining). All available epithelial and subepithelial areas from 2-3 biopsies of each subject were assessed and scored or counted. Depending on the size of the biopsies, more than 15 fields using x40 objective lens were scored for epithelial staining intensity for each subject. More than 1.5 mm² subepithelial area was measured and counted for MET+ and MMP10+ inflammatory cells for each subject. Cell counts were expressed as the number of cut cell profiles with a visible nucleus per mm² of the subepithelium.

Microarray mRNA analysis

Expression profiling was performed using Affymetrix U133 Plus 2.0 microarray (Affymetrix, Santa Clara, Calif) on total RNA extracted from bronchial biopsies. RNA purity (RIN >9.5) was measured by Agilent Bioanalyser (Agilent, Santa Clara, Calif). Raw data were quality-assessed and pre-processed by robust multi-array average normalization using Almac Pipeline and Pre-processing Toolbox (Almac, Craigavon, United Kingdom) and batch/technical effects were adjusted as covariates using general linear model.

Gene Set Variation Analysis (GSVA) and Transcription Factor Binding Sites Analysis

To facilitate biological interpretation, functionally-related genes were grouped as a gene set and their degree of expression summarized by GSVA (Bioconductor R package GSVA) as a quantitative enrichment score for each patient as previously described(11). In order to analyse for transcription factor (TF) binding sites, PRIMA (PRomoter Integration in Microarray Analysis) algorithm was used as previously described(12). Briefly, the algorithm used a position weighted matrices-based model to scan the promoter region of the target genes and identified the hits of putative binding sites of each TF. In turn, the hits of each TF from the target genes were determined for their over-representation with respect to a genome-wide background setting.

Statistical analysis

The datasets for this analysis were uploaded from the tranSMART system, an open-source knowledge management platform for sharing research data(13) supported by the European Translational Information and Knowledge Management Services (eTRIKS) project. All categorical variables were analyzed using Fisher's exact test. Student's t test was used for continuous variables with normal distribution, otherwise Wilcoxon rank-sum test was used. A linear model for microarray data (Bioconductor R package limma) with false discovery rate (FDR) correction was used for differential expression analysis. All p-values <0.05 were considered statistically significant.

Results

Transcriptomic profile in asthmatic bronchial biopsies

We first determined differences in the transcriptome of bronchial biopsies between asthmatics (n=81) and normal controls (n=41). We extracted a set of 251 DEGs and used unbiased principle component analysis (PCA) using these DEGs from the asthmatic cohort to identify significant clinical profiles associated with the variance of the gene expression. Upon visual inspection, no single clinical profile could account for the major variation of the first and second PC in the asthmatics cohort including the severity of asthma (**Fig S1**). A similar result was seen with hierarchical clustering where two major clusters were identified with no significant enrichment of specific clinical profiles including asthma severity was noted (**Fig S2A**, Chi-square test $p=0.740$). However, there was a trend towards a significant difference in the submucosal eosinophil profile between the two clusters (**Fig S2B**, Wilcoxon sign-rank test $p=0.091$). Using GSVA, we also found that there was little difference in expression levels of activation gene signatures for neutrophils, macrophages and CD4⁺ T cells between the mucosal eosinophil-high and eosinophil-low patients (data not shown).

Transcriptomic biomarker of eosinophilic inflammation

Because the comparison of asthmatic to normal controls did not yield any satisfactory clinical profiles, we next focused on the transcriptomic differences in bronchial biopsies between

eosinophil-high and eosinophil-low. To this end, the K-mean clustering method was applied which yielded four homogeneous mucosal eosinophil clusters (low, medium-low, medium-high and high; **Fig S3A**), as determined by the within-group sum of squares, using the eosinophil counts in bronchial biopsies that have been previously reported in this cohort (10) (**Fig S3B**). The low and medium-low clusters were further aggregated as the low mucosal eosinophil (LE) group (n=48), and the medium-high and high clusters as the high mucosal eosinophil (HE) group (n=33). The HE cohort had higher eosinophil counts compared to the LE cohort (9.8 vs 1.1 counts/mm², $p = 2.2 \times 10^{-9}$). To define the transcriptomic biomarkers of asthmatic eosinophilic inflammation in bronchial biopsies, we analyzed the DEGs between the HE and LE groups. Upon multiple testing correction with FDR, two probe sets at 205680_at (log2FC:1.31, adjusted-p value=0.035; MMP10) and 211599_x_at (log2FC:0.42, adjusted-p=0.035; MET) were found to be significantly expressed in the HE group (**Fig 1A**). There was a significant correlation between submucosal eosinophil counts and the expression of MMP10 (n=65; $r=0.549$; $p=2.23 \times 10^{-6}$), and of MET (n=65; $r=0.469$; $p=8.28 \times 10^{-5}$). Applying these two probe sets to differentiate high bronchial eosinophil inflammation using a support vector machine learning algorithm, a good classification performance was achieved as shown by ROC curve analysis (AUC: 0.853; 95%CI: 0.758-0.937, $p=3.97 \times 10^{-8}$; **Fig 1B**).

Activation of extracellular matrix gene pathway in HE group

In order to understand the biological pathway(s) underlying all potential genes characteristic of the HE group, we defined the DEGs from the HE group using a nominal level of significance at $p < 0.05$ and an absolute value of $\log_2$ fold change >0.5 . These genes were then subjected to an over-representation test for pathway analysis using three publicly available databases, GO, KEGG and Reactome. Overall, 73 genes were found to be characteristic of the HE group (**Fig 2A**), which corresponded to several top-ranked pathways including extracellular matrix (ECM) organization (GO:0030198; $p=2.2 \times 10^{-5}$, REAC:1474244; $p=1.1 \times 10^{-4}$) and regulation of leukocyte activation (GO:0002696; $p=0.0023$, **Table S2**). 33 out of the 73 genes were enriched in all the pathways identified, whereas 40 were left unannotated. Six functionally-relevant annotations were defined from the 33 genes, in which 10 genes including MMP10 were annotated as ECM organization, 4 as mast cell activation, 6 as C-C chemokine receptor binding, 5 as circulating immunoglobulin complex, 4 as serine proteinase inhibitors (SERPINs) and 5 as microtubule bundle formation (**Fig 2A** & **Table S3**). The expression of this gene signature was not different between those on oral corticosteroid treatment compared to those who were not.

We next used gene-set variation analysis (GSVA) to summarize each of the 6 upregulated functional annotated pathways into a signature score (i.e. enrichment score) for each subject, thus enabling a quantitative analysis of mutual signature relationships across all asthma participants. Using clustering analysis (**Fig 2B**), we showed that ECM, mast cell and C-C chemokine were the 3 parallel signatures, as they presented strong inter-signature relationships with high correlations noted between the ECM and mast cell signature (Pearson's $r=0.77$, $p=4.4 \times 10^{-17}$; **Fig 2C**) and between the ECM and C-C chemokine signature (Pearson's $r=0.68$, $p=2.9 \times 10^{-12}$; **Fig 2D**).

Implication of eosinophilic inflammation on severe and mild/moderate asthmatics

We next determined the molecular implications of a high degree of eosinophil infiltration in bronchial biopsies with the severity level. The molecular findings are in line with the pathological and clinical profiles of the HE group which showed significantly higher submucosal thickness of bronchial biopsies (9.6 vs. 8.2 μm , $p=0.006$), as well as peripheral blood eosinophilia (0.26 vs. 0.15 $\times 10^3/\mu\text{L}$, $p=0.005$) as compared to the LE group. In contrast, there was no difference in the proportion of patients with severe asthma between the HE and LE groups (60.6 vs. 62.5%, $p=1.000$, **Table S4**).

We therefore stratified these asthma patients by severity and analyzed whether there were differential effects of eosinophilic inflammation in severe versus non-severe asthma. In severe asthmatic subjects, HE showed significantly higher submucosal thickness (10.0 vs. 8.0 μ m, $p=0.019$), peripheral blood eosinophilia (0.30 vs. 0.19 $\times 10^3/\mu$ L, $p=0.026$), a trend toward lower FEV₁ predicted (64.9 vs. 70.5%, $p=0.076$) and frequency of atopy (55.0 vs. 83.3%, $p=0.062$, **Table 1**) as compared to the LE group. In mild/moderate asthmatic patients, the significantly higher submucosal thickness of bronchial biopsies was not seen in the HE group (9.1 vs. 8.3 μ m, $p=0.302$), whereas the trend towards peripheral blood eosinophilia (0.24 vs. 0.10 $\times 10^3/\mu$ L, $p=0.072$, **Table 2**) remained. In addition, the FeNO level was significantly higher in the HE (34.0 vs. 19.0 ppb, $p=0.029$, **Table 2**) than in the LE group, a finding which was not seen in severe asthma.

Pathway analysis of HE group for severe and mild/moderate asthmatics

We further analyzed the DEGs between HE and LE groups by analyzing the underlying biological pathways distinguishing the HE and LE groups in severe and mild/moderate asthmatics independently. In severe asthma patients, a total 66 genes were identified, including MMP10, CCL26 and POSTN which were all upregulated, and pathway enrichment analysis showed the top-ranked pathways as ECM organization (REAC:1474244; $p=0.003$) axonemal dynein complex assembly (GO:0070286; $p=0.007$) and microtubule bundle

formation (GO:0001578; $p=0.015$, **Table S5**). In contrast, in mild/moderate asthma subjects, a total 259 genes were identified, including upregulation of SOX7, IL33, TSLP and MMP10, and the biological pathways also revealed the enrichment in ECM organization (REAC:1474244; $p=9.2 \times 10^{-8}$), collagen biosynthesis (REAC:1650814; $p=1.3 \times 10^{-5}$), cell migration (GO:0016477; $p=2.5 \times 10^{-5}$) and interleukin-7 signaling (REAC:449147; $p=9.0 \times 10^{-5}$, **Table S5**).

Transcription factor binding site analysis for MMP10 and MET

Because the ECM signature, with MMP10 as its most highly-expressed gene, drove the biomarker and functional pathway analysis, we explored the regulation of these genes within the ECM signature by analysing associated transcription factors. To this end, we applied a position weighted matrices-based model to scan the promoter region (2000bp up- and 200bp downstream of transcription start site) of the 8 upregulated ECM genes. A set of 26 putative transcription factors (TFs) were identified whose binding sites were significantly enriched in the 8 gene promoters. We specifically focused on the 16 TFs (**Table S6**) that had binding sites at the MMP10 promoter and interrogated whether their gene expression was correlated with the expression of MMP10 as well as other ECM genes. Among the 16 TFs analyzed, we confirmed the correlation of the TF ETS1 gene with MMP10 (Pearson's $r=0.44$, $p=4.0 \times 10^{-5}$; **Fig S4A**) and with CTSG (Pearson's $r=0.32$, $p=0.0036$; **Fig S4B**) as well as a negative

relationship of NKX3A gene with MMP10 (Pearson's $r = -0.32$, $p = 0.0036$; **Fig S4C**). Three TFs (SOX, GATA6 and HNF4) were enriched at the MET promoter site but only the SOX family of TFs was significantly correlated with MET expression (**Fig S4D**).

Immunohistochemical localization of MET and MMP-10

In order to examine the potential role of MMP10 and MET in airway wall remodeling in asthma, we performed immunohistochemical analysis of these proteins in bronchial biopsies. Bronchial cells including basal epithelial cells, subepithelial polymorph and mononuclear inflammatory cells, fibroblasts and endothelial cells were stained positively and diffusely for both MET and MMP10. MET and MMP10 were expressed constitutively in the bronchial mucosa of normal controls (**Fig. 3A and B**) and were more highly expressed in asthmatics (**Fig. 3C and D**), respectively. The scores of epithelial MET and MMP10 positivity were higher in asthmatic than that in healthy subjects ($P = 0.042$ and 0.0017 , **Fig. 4A and B**, respectively). Counts of sub-epithelial MET- and MMP10-positive inflammatory cells trended towards significance and were significantly greater in asthma compared to normal ($P = 0.073$ and 0.042 , **Fig. 4C and D**, respectively).

Discussion

We found 73 differentially-expressed genes that were characteristic of high bronchial tissue eosinophilic asthmatics compared to the low tissue eosinophilic group. Pathway analysis was driven by 33 of these 73 genes and defined ECM organization, mast cell activation, CC-chemokine receptor binding, circulating immunoglobulin complex, serine protease inhibitors and microtubule bundle formation as key pathways. These pathways were associated with eosinophilic airway inflammation, increased blood and tissue macrophages, low systemic C-reactive protein levels and increased basement membrane thickness. *MMP10* and *MET* were the only genes which differentiated low from high eosinophilic inflammation in biopsies when more stringent criteria were applied and both *MET* and *MMP10* proteins were more highly expressed in airway epithelial cells and some submucosal inflammatory cells in asthma.

In our study, we used pathway analysis to highlight previously-identified aspects of asthmatic inflammation in bronchial biopsies. Mast cells are seen more commonly in airway smooth bundles of patients with asthma compared to healthy non-asthmatics (14); furthermore, eosinophils can produce the profibrogenic cytokines, TGF β and b-FGF-2, and also serine proteases (15). Mast cells may have an effect on airway wall remodeling, smooth muscle hypertrophy and mucus hypersecretion (16). It is also clear that CC-chemokines produced by epithelial cells and mast cells can not only recruit and activate infiltrating

immune cells but can also contribute to airway wall remodeling by inducing the proliferation of airway smooth muscle cells and activate the release of profibrogenic cytokines on human lung fibroblasts (17, 18). Our results also support a role for submucosal eosinophils in terms of extracellular matrix organization and mast cell activation, suggesting that there may be bi-directional interactions between eosinophils and mast cells. Mast cells are found in close proximity to eosinophils and physical interactions between those cells have been reported (19, 20).

Both MMP10 and MET have been implicated in lung cancer pathogenesis. MMP10 represents a critical lung cancer stem cell gene (21), while MET is a proto-oncogene (22). The product of MET is hepatocyte growth factor receptor (HGFR) and its ligand, hepatocyte growth factor (HGF), that upon binding to its receptor leads to receptor dimerisation and phosphorylation of its tyrosine kinase domain resulting in activation of important pathways that have been implicated in asthma such as PI3K-Akt signaling, Ras-MAP kinase cascade, STAT 3 and NF- κ B complex. These cascades can promote a variety of cellular function including proliferation and protection from apoptosis (23, 24). These signal transduction pathways have an important role in wound repair and hepatic regeneration, with an important role in liver development (25), raising a potential role in airway wall remodeling. HGF suppresses Th1 and Th2 immune responses (26) and induces secretion of MIP-1 β , MIP-2 α , IL6, IL8 and IL10 in monocytes (27, 28). The emerging role of MET in mediating

epithelial-mesenchymal transition, has received increasing attention with studies revealing the plasticity of epithelial cells with transformation into myofibroblasts that in turn produces excessive amount of extracellular matrix and collagen that contributes to airway fibrosis (29, 30). This transition may involve MET-HGF signaling cascades (31, 32).

In an integrative genomic analysis of airflow obstruction, pathway enrichment analysis led to the identification of several biologic modules including extracellular matrix processes, with MMP-10 as a candidate gene (33). The protective role of MMP-10 in the development of cigarette smoke-induced emphysema in a knock-out mouse model has also been reported (33), but its role in asthma remains to be determined. MMP10 is upregulated in small airways in COPD (34). MMP10 gene expression is upregulated in airway epithelial cells infected with rhinovirus (35) and respiratory syncytial virus (36). MMP10 may drive the conversion of pro-inflammatory M1-biased macrophages towards immunosuppressive M2-biased cells, and, in the context of airway remodelling, MMP-10 also controls the activation of ECM-degrading activity in M2 macrophages (37).

In the analysis of the regulatory factors for MMP-10 and MET, two transcription factors, ETS-1 and SOX (SRY-related HMG-box) family proteins were identified. SOX proteins are involved in processes such as apoptosis, tumorigenesis and remodelling (38) and as such, may be implicated in airway remodelling in severe eosinophilic asthma. SOX4, 7 and 15 are part of the human large airway basal cell gene signature (39). SOX4 has also been

implicated in lung epithelial cell-mediated host defence against bacteria by regulating the expression of antimicrobial factors such as lysozyme (40). Furthermore, SOX7 is involved in basal keratinocyte function and in epidermal-dermal junction plasticity and remodelling in the skin (41). Our data highlighted an inverse correlation between ETS1 and NKX3A, and MMP10 expression. ETS1 is associated with up-regulation of pro-fibrotic genes controlled by the TGF β -signalling pathway in models of lung fibrosis (42), and mediates TNF α -induced MMP9 and tenascin expression in primary human bronchial fibroblasts, thus potentially playing a key role in airway remodeling (43). ETS1 may also drive VEGF-induced MMP10 expression and endothelial cell migration and tube formation in vitro (44), suggesting that the ETS1/MMP10 axis may also be involved in the angiogenesis of severe asthma (45). ETS1 may also have a role in the regulation of MET expression (46). Finally, ETS1 may provide a link between eosinophilia and remodelling as ETS1 can co-operate with GATA3 to induce IL-5 expression in human Jurkat T-cells (47) and regulates GM-CSF production from human mast cells (48). NKX3A is a homeobox-containing transcription factor that acts as a negative regulator of epithelial cell growth in prostate tissue and is a major driver of prostate cancer (49). Future studies should examine the functional effects of ETS1 and SOX4, 7 and 15 on MMP10 and MET expression in primary basal airway epithelial cells and in animal models of severe eosinophilic asthma.

One of the strengths of the study has been the ability to relate mucosal eosinophils with its transcriptomic profile in order to attempt to define the role of the eosinophil in the remodeling process. However, there are some potential limitations of the data. First, there was relatively lower levels of mucosal eosinophil counts found in the asthmatic subjects compared to other similar cohorts (50, 51), but this could be explained by the high dose inhaled corticotherapy with a high proportion on daily oral corticosteroid therapy. Secondly, there was a relatively high level of eosinophil counts in the control non-asthmatic subjects that may be related to their level of atopic background; however, we did not use the comparison of asthmatic subjects to the control subjects because the hierarchical analysis did not yield significant differentiation of genes. Finally, we have not been able to examine the potential contribution of other inflammatory cells apart from eosinophils that could contribute to the complex process of airway wall remodeling. However, using GSVA, we found that there was little difference in expression levels of activation gene signatures for neutrophils, macrophages and CD4⁺ T cells between the mucosal eosinophil-high and eosinophil-low patients (data not shown). A final limitation of our study is that we have not been able to validate our findings in an independent cohort, including a functional validation of MMP10 and MET in severe asthma.

In summary, asthma characterized by submucosal airway eosinophilic inflammation is associated with increased subbasement membrane thickness with *MMP-10* and *MET* identified as most differentially-expressed genes, and their regulatory factors comprises ETS-1 and SOX. These findings indicate the potential involvement of these factors underlying the submucosal eosinophilic inflammation and subepithelial fibrosis observed in asthma that warrant further investigation.

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Author Contributions:

CSK, SP, ML and FB performed the analysis; KFC, IMA, AR, KS, CA and YG designed the analytical approaches taken and analyzed the results; DG, UH, PH, DS, SF, BD, PC, NK, TS,

LF, PHH, MC, LF, SJW and BD participated in the clinical characterization of the patients; IP, AS and JC were part of the data curation team; IMA, RD, PJS, and KFC conceived of and designed the study; and KFC, IMA & CSK drafted the manuscript.

Conflict of interest statement

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Table 1: Comparison of clinical features and submucosal cell profiles of the HE vs LE group in severe asthmatics

Variables	HE (n=20)	LE (n=30)	Missing data, n (%)	p-value
Age (years)	52.2±12.7	51.4±11.4	-	0.814
Female	11 (55.0)	14 (46.7)	-	0.772
BMI	28.6±5.2	31.2±6.5	-	0.115
Smoking	4 (20.0)	10 (33.3)	-	0.479
Nasal polyp	6 (30.0)	12 (40.0)	-	0.673
Allergic rhinitis	12 (60.0)	13 (43.3)	-	0.386
Eczema	9 (45.0)	15 (50.0)	-	0.954
Oral corticosteroid use	10 (50.0)	12 (40.0)	-	0.684
Atopy	11 (55.0)	25 (83.3)	-	0.062
Exacerbations (per year)	3.0 (2.0-4.0)	2.5 (1.8-3.0)	14 (28.0)	0.603
FEV ₁ (% predicted)	64.9 (50.8-74.1)	70.5 (57.5-87.6)	-	0.076
Total serum IgE (IU/ml)	179.0 (68.1-458.5)	85.9 (31.7-351.0)	1 (2.0)	0.218
Blood eosinophil (10 ³ /μl)	0.30 (0.20-0.40)	0.19 (0.10-0.29)	-	0.026
Sputum eosinophil (%)	6.1 (0.7-23.9)	2.1 (0.2-14.3)	25 (50.0)	0.443
FeNO (ppb)	30.0 (18.3-49.0)	26.0 (17.0-47.0)	5 (10.0)	0.794
Serum periostin (ng/ml)	45.2 (41.9-52.8)	44.0 (38.9-53.7)	7 (14.0)	0.605
Serum CRP (mg/l)	1.5 (1.0-4.2)	5.0 (1.1-7.0)	1 (2.0)	0.094
<i>Submucosal cell counts</i>				
Eosinophil (mm ⁻³)	7.8 (5.7-13.4)	1.0 (0-2.2)	-	2.3x10 ⁻⁹
Neutrophil (mm ⁻³)	16.0 (6.3-22.2)	12.1 (8.4-17.1)	-	0.294
Macrophage (mm ⁻³)	4.1 (2.2-8.6)	2.0 (0.7-3.3)	-	0.005
CD4 T cell (mm ⁻³)	12.2 (7.8-15.4)	6.5 (4.3-11.8)	-	0.070
CD8 T cell (mm ⁻³)	20.7 (13.5-34.7)	12.7 (4.6-22.5)	-	0.099
Mast cell (mm ⁻³)	20.8 (15.8-34.1)	13.6 (4.6-23.3)	-	0.111
ASM fraction (%)	0.33 (0.26-0.39)	0.29 (0.22-0.42)	-	0.642
Thickness (μm)	10.0 (8.7-10.7)	8.0 (7.0-9.8)	9 (18.0)	0.019

Data presented as N (%) and mean (SD) or median (IQR). ASM: Airway smooth muscle; BMI: Body mass index; CRP: C-reactive protein; EOS: eosinophil; FeNO: Fractional exhaled nitric oxide; FEV₁: Forced expiratory volume in 1 second; IgE: Immunoglobulin E.

Table 2: Comparison of clinical features and submucosal cell profiles of the HE vs LE group in mild/moderate asthmatics

Variables	HE (n=13)	LE (n=18)	Missing data, n (%)	p-value
Age (years)	38.9±10.7	41.2±15.4	-	0.617
Female	4 (30.8)	12 (66.7)	-	0.107
BMI	26.4±4.7	27.7±6.3	-	0.412
Smoking	0	0	-	-
Nasal polyp	0	2 (11.1)	-	0.616
Allergic rhinitis	7 (53.8)	8 (44.4)	-	0.879
Eczema	3 (23.1)	5 (27.8)	-	1.000
Atopy	10 (76.9)	14 (77.8)	-	1.000
Exacerbations (per year)	0	1.0 (1.0-1.0)	25 (80.6)	0.905
FEV ₁ (% predicted)	95.2 (73.5-104.0)	92.8 (72.2-102.0)	-	0.708
Total serum IgE (IU/ml)	125.0 (23.0-196.0)	58.8 (44.3-121.8)	-	0.378
Blood eosinophil (10 ³ /μl)	0.24 (0.20-0.30)	0.10 (0.10-0.25)	-	0.072
Sputum eosinophil (%)	0.45 (0.14-1.21)	0 (0-0.57)	18 (58.1)	0.498
FeNO (ppb)	34.0 (22.0-57.0)	19.0 (12.9-21.8)	-	0.029
Serum periostin (ng/ml)	44.0 (37.3-51.3)	43.5 (39.4-47.8)	4 (12.9)	0.683
Serum CRP (mg/l)	1.0 (1.0-2.3)	2.0 (1.0-5.0)	3 (9.7)	0.253
<i>Submucosal cell counts</i>				
Eosinophil (mm ⁻³)	6.1 (5.2-11.1)	0.7 (0-1.4)	-	2.7x10 ⁻⁶
Neutrophil (mm ⁻³)	11.1 (10.6-18.9)	9.1 (5.1-13.0)	-	0.230
Macrophage (mm ⁻³)	4.0 (2.2-7.3)	1.6 (0.7-3.5)	-	0.043
CD4 T cell (mm ⁻³)	14.8 (9.0-35.2)	9.1 (1.1-11.6)	-	0.012
CD8 T cell (mm ⁻³)	22.0 (13.6-35.4)	11.9 (5.0-19.3)	-	0.082
Mast cell (mm ⁻³)	29.0 (21.1-37.6)	19.6 (16.2-36.0)	-	0.246
ASM fraction (%)	0.30 (0.25-0.32)	0.35 (0.25-0.47)	-	0.367
Thickness (μm)	9.1 (8.0-9.7)	8.3 (7.0-9.3)	4 (12.9)	0.302

Data presented as N (%) and mean (SD) or median (IQR). ASM: Airway smooth muscle; BMI: Body mass index; CRP: C-reactive protein; EOS: eosinophil; FeNO: Fractional exhaled nitric oxide; FEV₁: Forced expiratory volume in 1 second; IgE: Immunoglobulin E.

Figure legends

Figure 1: Biomarker analysis for mucosal eosinophilic inflammation. (A) Volcano plot showing probe set/transcript level expression comparing the HE against LE group, where x-axis is $\log_2$ fold-change, and y-axis is $-\log$ p-value adjusted for FDR. At $\text{FDR} < 0.05$ (red dashed line), two probe sets as 205680_at ($\log_2\text{FC}:1.31$, adjusted- $p=0.035$, *MMP10*) and 211599_x_at ($\log_2\text{FC}:0.42$, adjusted- $p=0.035$, *MET*) were differentially expressed. (B) Receiver operated characteristic (ROC) curve showing good performance of *MMP10* and *MET* probe sets as classifiers of mucosal eosinophilic inflammation using the support vector machine learning algorithm.

Figure 2: Functional/biological annotation of the genes characteristic of mucosal eosinophilic inflammation. (A) Volcano plot showing gene level expression comparing the HE vs. LE group (black dashed line: absolute value of $\log_2$ fold change > 0.5 , red dashed line: nominal $p < 0.05$). 73 genes were identified characteristic of the HE group, of which 10 were annotated as extracellular matrix (ECM, red) organization, 4 as mast cell activation (black), 6 as C-C chemokine receptor binding (green), 5 as circulating immunoglobulin complex (Ig, yellow), 4 as serine proteinase inhibitors (SERPINs, magenta) and 5 as microtubule bundle formation (MT, orange). (B) Heat map showed clustering of 6 GSVA signatures at row side and column side corresponding to subjects of LE group (grey bar) and HE group (magenta bar). (C) High correlation of GSVA signatures were identified between ECM and mast cell gene sets (Pearson's $r=0.77$, $p=4.4 \times 10^{-17}$) and (D) between ECM and C-C chemokine gene sets (Pearson's $r=0.68$, $p=2.9 \times 10^{-12}$).

Figure 3. Immunohistochemistry for MET and MMP-10 in bronchial biopsy sections. There is weaker immunostaining and fewer subepithelial positive cells in a normal subject compared to an asthmatic subject for both MET (A and C for normal and asthmatic, respectively) and MMP-10 (B and D for normal and asthmatic, respectively) (E) shows negative control with absence of signal. Internal scale bar = 20 μm for all panels. Figures are representative of staining of samples from 6 healthy non-asthmatic (normal) subjects and 10 asthmatic subjects.

Figure 4. Scores of epithelial staining intensity for MET (A) and MMP10 (B) in bronchial biopsies of normal non-asthmatic ($\triangle$) and asthmatic subjects ($\blacktriangle$). Counts for subepithelial MET⁺ (C) and MMP10⁺ (D) cells in bronchial biopsies of normal and asthmatic subjects. Data are expressed as the number of positive cells per mm^2 of subepithelium. Individual scores and counts are shown with horizontal bars showing median values.

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Fig 3

Fig 4