



# Severe asthma exists despite suppressed tissue inflammation: findings of the U-BIOPRED study

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**ABSTRACT** The U-BIOPRED study is a multicentre European study aimed at a better understanding of severe asthma. It included three steroid-treated adult asthma groups (severe nonsmokers (SAn group), severe current/ex-smokers (SAs/ex group) and those with mild-moderate disease (MMA group)) and healthy controls (HC group). The aim of this cross-sectional, bronchoscopy substudy was to compare bronchial immunopathology between these groups.

In 158 participants, bronchial biopsies and bronchial epithelial brushings were collected for immunopathologic and transcriptomic analysis. Immunohistochemical analysis of glycol methacrylate resin-embedded biopsies showed there were more mast cells in submucosa of the HC group ( $33.6 \text{ mm}^{-2}$ ) compared with both severe asthma groups (SAn:  $17.4 \text{ mm}^{-2}$ ,  $p < 0.001$ ; SAs/ex:  $22.2 \text{ mm}^{-2}$ ,  $p = 0.01$ ) and with the MMA group ( $21.2 \text{ mm}^{-2}$ ,  $p = 0.01$ ). The number of  $\text{CD4}^{+}$  lymphocytes was decreased in the SAs/ex group ( $4.7 \text{ mm}^{-2}$ ) compared with the SAn ( $11.6 \text{ mm}^{-2}$ ,  $p = 0.002$ ), MMA ( $10.1 \text{ mm}^{-2}$ ,  $p = 0.008$ ) and HC ( $10.6 \text{ mm}^{-2}$ ,  $p < 0.001$ ) groups. No other differences were observed.

Affymetrix microarray analysis identified seven probe sets in the bronchial brushing samples that had a positive relationship with submucosal eosinophils. These mapped to *COX-2* (cyclo-oxygenase-2), *ADAM-7* (disintegrin and metalloproteinase domain-containing protein 7), *SLCO1A2* (solute carrier organic anion transporter family member 1A2), *TMEFF2* (transmembrane protein with epidermal growth factor like and two follistatin like domains 2) and *TRPM-1* (transient receptor potential cation channel subfamily M member 1); the remaining two are unnamed.

We conclude that in nonsmoking and smoking patients on currently recommended therapy, severe asthma exists despite suppressed tissue inflammation within the proximal airway wall.



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## Introduction

Asthma is a heterogeneous disorder that varies in severity and response to treatment. Many different cell populations are involved, including structural airway cells as well as infiltrating leukocytes [1]. To date, however, there are no pathological markers that distinguish severe asthma from less severe disease, although some inflammatory and remodelling features are reported to have greater expression. These include increased neutrophil numbers in sputum [2], biopsies and bronchoalveolar lavage fluid [3, 4], an increase in the area of airway smooth muscle and glands [4–6], as well as increased thickness of the lamina reticularis [4, 7]. However, these observations are not unanimous, with some publications identifying no increase in neutrophils [5] or in the thickness of the lamina reticularis [5, 6] in severe asthma compared with mild or moderate asthma. These discrepancies may reflect the range of clinical and pathological phenotypes described in asthma [7–12], and the inclusion of these differing populations.

Severe asthma has been divided into eosinophilic and noneosinophilic phenotypes when airway inflammation is assessed in sputum or endobronchial biopsies [4, 8, 9, 13–16]. In severe asthma with bronchial mucosal eosinophilia, there are also increased numbers of airway lymphocytes, mast cells and macrophages, together with increased thickness of the lamina reticularis [13, 16]. These findings differ from those in severe asthma without eosinophils but not in mild asthma or healthy individuals [13]. Those with eosinophilic disease are the most responsive to steroid therapy [16, 17], although in severe asthma airway eosinophilia can persist despite such treatment. Not all severe asthma is, however, eosinophilic [4, 13–15].

Whilst previously there have been several large multicentre studies of severe asthma, including the two European studies ENFUMOSA (European network for understanding mechanisms of severe asthma) and BIOAIR (Longitudinal Assessment of Clinical Course and Biomarkers in Severe Chronic Airway Disease), and the SARP (Severe Asthma Research Program) study in the USA [18], there is still a need to better understand the basis for disease persistence and inflammatory phenotypes in severe asthma to permit the stratified application of novel therapies. Towards this end, the U-BIOPRED (Unbiased Biomarkers for the Predictions of Respiratory Disease Outcomes) consortium aimed to subphenotype severe asthma using an innovative systems medicine approach. This comprehensive study included a variety of “omics” technologies applied to bronchoscopic airway, sputum and blood samples, noninvasive sampling with exhaled breath measures, together with clinical and pathological characterisation conducted using the same standard operating procedures (SOPs) across all centres. The aim of the study reported here was to investigate the immunopathology in the airways of severe asthma subjects as compared with mild–moderate asthma subjects and healthy controls. In addition, we examined the association of smoking with airway pathology in severe asthma. We also looked at the influence of stratification by eosinophilia and neutrophilia, and the relationship of this to gene expression.

## Methods

### Study design

The U-BIOPRED study is a multicentre study involving 20 academic centres in 11 European countries, 11 pharma partners and six patient organisations. Three steroid-treated adult asthma groups (severe nonsmokers (SAn group), severe current/ex-smokers (SAs/ex group) and those with mild–moderate disease (MMA group)), classified and treated according to the Global Initiative for Asthma guidelines, as well as healthy controls (HC group) were recruited in the main U-BIOPRED study. Definitions for each group were agreed at a consensus meeting prior to study start, and are described in full by SHAW *et al.* [19] and in the online supplementary material. At baseline, participants underwent clinical screening to assess demographics, comorbidities, asthma symptoms, asthma history, treatment and lung function. Induced sputum and blood samples were collected for assessment of inflammatory cell profile and “omics” analysis with measures of the exhaled nitric oxide fraction ( $F_{eNO}$ ).

Eight of the clinical centres took part in a cross-sectional bronchoscopy substudy. An ethics review committee approved the study in each of these centres and all study volunteers gave written informed consent.

#### ***Bronchoscopy procedure and collection of samples***

Participants undergoing bronchoscopy had to meet the inclusion criteria for the main study [19] and none of the bronchoscopy exclusion criteria (see online supplementary material). All the participating centres followed SOPs for the bronchoscopy, airway sampling and processing (see online supplementary material).

Up to eight proximal airway endobronchial biopsies were collected from each subject: three for immunohistochemistry (processed into glycol methacrylate resin (GMA)), three for transcriptomics (placed into RNAlater stabilising reagent), and up to two biopsies fixed in 10% neutral buffered formalin and embedded in paraffin wax for biobanking for future use. Biopsies were initially processed at the clinical centres according to the SOPs, with each centre having received training and undergone a pre-study biopsy quality control assessment, before shipping to a central laboratory for analysis or to the centralised biobank for storage in accordance with international guidelines. Epithelial brushings were collected from the proximal airways using disposable, sheathed bronchial brushes and placed in PBS for processing for transcriptomics.

#### ***Bronchial biopsy immunohistochemical analysis***

Immunohistochemical analysis of the GMA resin-embedded biopsies was undertaken, as previously described [20], in the Histochemistry Research Unit at the University of Southampton (Southampton, UK). Only biopsies passing defined quality criteria (see online supplementary material) were immunohistochemically stained for the presence of mast cells, eosinophils, neutrophils and macrophages, as well as CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> and CD25<sup>+</sup> lymphocytes. Sections were also stained to identify airway smooth muscle (ASM).

Positive nucleated cells were enumerated as cells per square millimetre of submucosa and ASM, and per millimetre length of epithelium. This was derived by counting cells in the submucosa, ASM and epithelium in each section, calculating the mean number for each cell type for each subject, and measuring the area of the submucosa and ASM; epithelial length was determined using a calibrated image analysis system. The thickness of the lamina reticularis was also assessed with the assistance of computerised image analysis [21]. A point counting grid was used on the sections stained for  $\alpha$ -smooth muscle actin to determine the muscle proportion, *i.e.* the volume fraction (proportion) [22]. SOPs for these methods are in the online supplementary material.

#### ***Transcriptomic analysis of biopsies and brushings***

The RNAlater samples were shipped to a single site *via* the biobank for assessment of RNA quality, transcriptomic processing and analysis. RNA was isolated from the samples using a miRNeasy kit (Qiagen, Germantown, MD, USA) and amplified with a Ovation Pico WTA kit (NuGen Technologies, San Carlos, CA, USA). The cDNA was analysed using a HT HG-U133+ PM microarray platform (Affymetrix, Santa Clara, CA, USA). Quality checks were performed following Affymetrix's recommendations and post-analysis (see online supplementary material for further details).

#### ***Data analysis***

Initial analysis of immunohistochemical, sputum, blood, exhaled air and clinical data was by ANOVA to test for differences between groups, and then where relevant either nonparametric or parametric analysis was applied to evaluate the significance of group differences using SPSS version 19 (IBM, Armonk, NY, USA). Spearman's rank test was applied to test for pairwise correlation.

The background-corrected, normalised transcriptomic data for the bronchial biopsies and bronchial brushings were tested for association with submucosal eosinophil and neutrophil counts using a general linear model, adjusting for covariates age and gender, and correction for false discovery rate (see online supplementary material for further details). Analyses were conducted using R version 3.2.2 ([www.r-project.org](http://www.r-project.org)).

#### ***Stratification by eosinophil and neutrophil number***

To investigate the relevance of eosinophilia and neutrophilia to immunohistochemical, blood and sputum cell count data, and FeNO, as well as clinical characteristics, data were divided into low and high groups based on the mean+2SD eosinophil and neutrophil submucosal count in the HC group [12].

### **Results**

A total of 160 participants underwent bronchoscopy, two of which were withdrawn from the study due to protocol violations. Of the remaining 158, biopsies suitable for immunohistochemistry were obtained from 87% of the participants (n=137) with a mean of 1.4 suitable biopsies per participant. These comprised 46

in the SAn group, 16 in the SAs/ex group, 34 in the MMA group and 41 in the HC group. The baseline clinical data and inflammatory cell profiles in the induced sputum and blood are summarised in table 1, and the biopsy data are summarised in table 2. The four groups for ANOVA ranged in size from 16 to 46. Under the simplifying assumption of equal group sizes ( $n=39$ ), there is 15%, 74% and >95% power to detect small, medium and large effect sizes, respectively, at the 5% level. The three effect sizes were parameterised by Cohen's  $f$ , taking accepted values of 0.1, 0.25 and 0.4, respectively [23]. It is acknowledged that some small or moderate effects are likely to have been missed.

### Participant demographics

The bronchoscopy subgroup demographics were similar to the overall study [19]. The participants in both severe asthma (SAn and SAs/ex) groups were older, had a higher body mass index, lower forced expiratory volume in 1 s (FEV<sub>1</sub>), more exacerbations and increased prevalence of nasal polyps than the MMA and HC groups. Atopy and rhinitis were increased compared with the HC group but not the MMA group. There was more gastro-oesophageal reflux disease in the SAn group compared with the MMA group and rhinitis was more prevalent in the SAn group compared with the SAs/ex group.

TABLE 1 Subject characteristics: bronchoscopy cohort

	Group			
	SAn	SAs/ex	MMA	HC
<b>Subjects</b>	46	16	34	41
<b>Age years</b> <sup>¶,*,§,f</sup>	50.5±2.1	52.0±2.1	39.5±2.4	39.9±2.1
<b>Sex</b>				
Female	24	7	19	13
Male	22	9	15	28
<b>BMI kg·m<sup>-2</sup></b> <sup>¶,*,§,f</sup>	30.2±0.9	30.1±1.5	26.5±0.8	25.2±0.6
<b>FEV<sub>1</sub> % pred</b> <sup>¶,*,§,f,##</sup>	73.0±3.1	64.7±3.6	93.1±2.9	102.0±1.8
<b>Exacerbations in last year</b> <sup>¶,§</sup>	2.8±0.3 (n=34)	2.9±0.4 (n=12)	1.4±0.4 (n=34)	NA
<b>Smoking history</b>				
Never-smoker	83		88	80
Current smoker		31		
Ex-smoker (<5 pack-years)	17	69	12	20
<b>Positive atopic status</b> <sup>¶,*,§,##</sup>	33/43 (77)	12/14 (86)	278/28 (96)	14/30 (47)
<b>Rhinitis diagnosed</b> <sup>¶,*,§,##</sup>	37/45 (82)	8/16 (50)	29/31 (94)	6/13 (46)
<b>Nasal polyps</b> <sup>¶,§</sup>	17/43 (40)	6/15 (40)	2/32 (6)	3/12 (25)
<b>GORD</b> <sup>¶</sup>	19/35 (54)	8/14 (57)	9/31 (29)	9/26 (35)
<b>Regular medication used</b>				
Anti-IgE	5/41 (12)	1/12 (8)	NA	NA
Antibiotics	12/43 (28)	5/11 (45)	NA	NA
LT modifier	26/44 (59)	7/14 (50)	NA	NA
Macrolides	8/46 (17)	7/15 (47)	NA	NA
OCS	19/44 (43)	7/14 (50)	NA	NA
Xanthines	7/42 (17)	1/12 (8)	NA	NA
<b>F<sub>ENO</sub> ppb</b> <sup>¶,*</sup>	38.0 (20.0–60.5) (n=41)	20.0 (12.5–34.3) (n=15)	22.0 (17.5–55.0) (n=34)	18.3 (14.5–29.0) (n=38)
<b>Blood eosinophils</b>				
Count ×10 <sup>3</sup> μL <sup>-1</sup> <sup>¶,*,§,##</sup>	0.3 (0.1–0.4)	0.2 (0.2–0.2)	0.2 (0.1–0.3)	0.1 (0.1–0.2)
%	3.6 (1.7–6.3)	1.8 (1.2–3.6)	3.1 (1.9–5.55)	2.3 (1.5–3.3)
<b>Blood neutrophils</b>				
Count ×10 <sup>3</sup> μL <sup>-1</sup> <sup>¶,*,§,f</sup>	4.8 (3.4–6.1)	6.84 (4.0–8.6)	3.2 (2.7–4.6)	2.7 (2.2–3.7)
% <sup>¶,*,§,f</sup>	63.1 (54.3–70.4)	63.9 (54.3–76.7)	56.6 (51.9–61.9)	55.9 (50.0–62.9)
<b>Sputum eosinophils</b>				
Count <sup>¶,*,f</sup>	15.0 (2.0–64.0)	36.0 (2.0–101.0)	3.5 (0–11.0)	0 (0–2.0)
% <sup>¶,*,f</sup>	2.8 (0.5–12.4)	7.1 (0.4–18.9)	0.6 (0–2.1)	0 (0–0.3)
<b>Sputum neutrophils</b>				
Count <sup>¶,*,##</sup>	292.0 (205.0–389.5)	223.5 (183.0–264.0)	258.0 (139.5–388.5)	139.5 (92.0–235.0)
% <sup>¶,*,##</sup>	52.7 (38.7–70.4)	39.4 (31.8–521)	52.2 (28.5–68.4)	28.5 (16.2–47.2)

Data are presented as n, mean±SE, %, n/N (%) or median (interquartile range), unless otherwise stated. If there is missing data the number of subjects data is available from is given. SAn: severe asthma nonsmokers; SAs/ex: severe asthma current/ex-smokers; MMA: mild-moderate asthma; HC: healthy controls; BMI: body mass index; FEV<sub>1</sub>: forced expiratory volume in 1 s; GORD: gastro-oesophageal reflux disease; LT: leukotriene; OCS: oral corticosteroid; F<sub>ENO</sub>: exhaled nitric oxide fraction. Differences ( $p<0.05$ ) are indicated as: <sup>¶</sup>: SAn versus SAs/ex; <sup>§</sup>: SAs/ex versus MMA; <sup>f</sup>: SAs/ex versus HC; <sup>##</sup>: MMA versus HC.

TABLE 2 Summary of biopsy immunopathology results

	Group			
	SAn	SAs/ex	MMA	HC
<b>Subjects</b>	46	16	34	41
<b>Samples analysed for each biopsy parameter</b>				
Submucosa	46	16	34	41
ASM	39	13	31	37
Epithelium	37	16	31	28
Lamina reticularis	37	14	30	28
ASM fraction	45	16	33	39
<b>Mast cells</b>				
Submucosa mm <sup>-2</sup> ¶,§,f	17.4 [8.7–27.1]	22.2 [1.1–35.8]	21.2 [16.3–34.1]	33.6 [25.9–43.3]
ASM mm <sup>-2</sup>	5.3 [0–13.8]	10.3 [0.8–16.7]	9.6 [3.7–26.4]	14.3 [2.6–22.3]
Epithelium mm <sup>-1</sup>	0 [0–1.9]	0 [0–0.4]	0 [0–2.3]	0 [0–1.6]
<b>Eosinophils</b>				
Submucosa mm <sup>-2</sup>	3.4 [1.1–7.6]	2.1 [0.8–5.0]	2.1 [0.6–5.6]	2.1 [0.6–4.9]
<b>Neutrophils</b>				
Submucosa mm <sup>-2</sup>	12.2 [4.8–20.7]	13.6 [8.7–18.6]	11.0 [6.7–14.1]	13.2 [6.3–22.1]
Epithelium mm <sup>-1</sup>	0.3 [0–2.4]	0.5 [0–1.2]	0 [0–1.0]	0 [0–1.4]
<b>Macrophages</b>				
Submucosa mm <sup>-2</sup>	2.2 [1.1–5.3]	2.1 [0.7–3.8]	3.0 [0.9–4.4]	2.2 [1.1–4.26]
<b>CD3<sup>+</sup> lymphocytes</b>				
Submucosa mm <sup>-2</sup>	35.8 [22.4–45.3]	20.6 [13.0–35.5]	35.9 [22.2–50.9]	36.8 [24.7–70.2]
Epithelium mm <sup>-1</sup>	1.5 [0–5.9]	2.2 [1.1–7.8]	1.7 [0–7.3]	2.9 [1.4–7.8]
<b>CD4<sup>+</sup> lymphocytes</b>				
Submucosa mm <sup>-2</sup> #,+,§	11.6 [5.1–18.8]	4.7 [2.9–6.9]	10.1 [4.0–19.1]	10.6 [7.6–22.3]
<b>CD8<sup>+</sup> lymphocytes</b>				
Submucosa mm <sup>-2</sup>	15.9 [5.3–30.0]	12.7 [5.2–21.4]	14.6 [7.1–26.4]	21.0 [11.8–37.8]
Epithelium mm <sup>-1</sup>	0.3 [0–2.6]	1.3 [0.9–4.8]	0.45 [0–3.8]	1.95 [0.6–4.5]
<b>Lamina reticularis thickness µm</b>	8.9 [7.3–10.2]	8.7 [7.9–9.9]	9.0 [7.7–9.7]	8.8 [8.0–9.2]
<b>ASM volume fraction</b>	0.3 [0.2–0.4]	0.3 [0.2–0.4]	0.3 [0.3–0.5]	0.3 [0.2–0.4]

Data are presented as n or median (interquartile range). Zero data is not shown. SAn: severe asthma nonsmokers; SAs/ex: severe asthma current/ex-smokers; MMA: mild-moderate asthma; HC: healthy controls; ASM: airway smooth muscle. Differences (p<0.05) are indicated as: #, SAn versus SAs/ex; ¶, SAn versus HC; \*, SAs/ex versus MMA; §, SAs/ex versus HC; f, MMA versus HC.

### Baseline inflammatory measures

A number of differences were detected between the four groups. The percentage of sputum eosinophils was higher in the SAn group than in the MMA group, and in both the SAn and SAs/ex groups than in the HC group. The percentage of sputum neutrophils was higher in the SAn and MMA groups than in the HC group. Neutrophils in the peripheral blood were higher in the SAs/ex group compared with the SAn group, and in both severe asthma groups (SAn and SAs/ex) were higher than in the MMA and HC groups. There was no difference in peripheral blood eosinophilia between the groups. FeNO was higher in the SAn group compared with the SAs/ex and HC groups.

### Immunopathology data

Differences were observed across the four groups in the number of submucosal mast cells (p=0.001) and submucosal CD4<sup>+</sup> lymphocytes (p=0.004). There were no other across-group differences. There were higher numbers of mast cells in the bronchial submucosa in the HC group, with a median of 33.6 mm<sup>-2</sup>, compared with all three asthma (SAn, SAs/ex and MMA) groups (figure 1a). Mast cell median numbers were 17.6 mm<sup>-2</sup> in the SAn (p<0.001), 22.2 mm<sup>-2</sup> in the SAs/ex (p=0.014) and 21.3 mm<sup>-2</sup> in the MMA (p=0.012) groups. There were significantly fewer CD4<sup>+</sup> lymphocytes in the submucosa of the SAs/ex group (4.7 mm<sup>-1</sup>) compared with the SAn (11.6 mm<sup>-2</sup>, p=0.001), MMA (10.1 mm<sup>-2</sup>, p=0.008) and HC (10.6 mm<sup>-2</sup>, p<0.001) groups (figure 1b).

A positive relationship, across all groups was observed between percentage blood eosinophils and submucosal eosinophils (rho=0.462, p<0.001), but there was no relationship with sputum eosinophils. In addition, both blood eosinophils and submucosal eosinophils correlated with the thickness of the lamina reticularis (rho=0.222, p=0.021 and rho=0.21, p=0.023, respectively). Furthermore, eosinophils in blood, submucosa and sputum correlated with FeNO (rho=0.325, p<0.001, rho=0.440, p<0.001 and rho=0.218, p=0.014, respectively) (figure 2). FEV<sub>1</sub> % pred had an inverse relationship with neutrophils in the blood

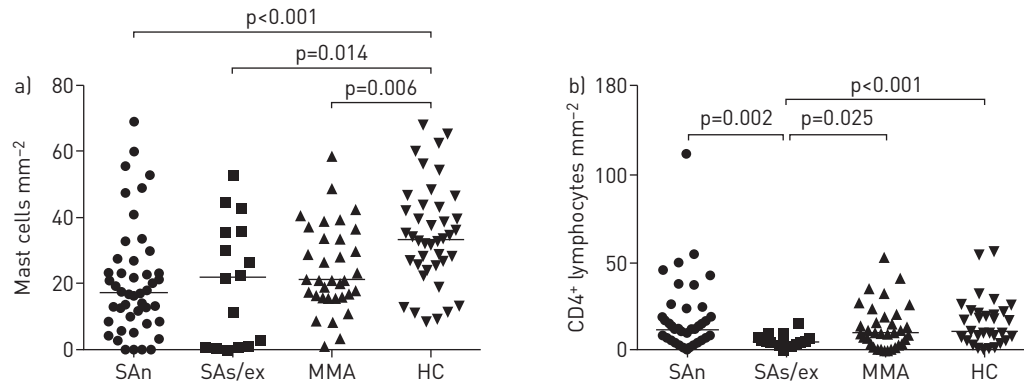


FIGURE 1 a) Mast cells and b) CD4<sup>+</sup> lymphocytes in the bronchial submucosa in severe asthma nonsmokers (SAn group), severe asthma current/ex-smokers (SAs/ex group), mild-moderate asthma (MMA group) and healthy controls (HC group). Median values and significant differences between the groups are indicated.

( $\rho = -0.253$ ,  $p = 0.003$ ), eosinophils and neutrophils in sputum ( $\rho = -0.394$ ,  $p = 0.001$  and  $\rho = -0.315$ ,  $p = 0.009$ , respectively) and FeNO ( $\rho = -0.197$ ,  $p = 0.026$ ), and a positive relationship with mast cells in the submucosa ( $\rho = 0.356$ ,  $p < 0.001$ ) (figure 3).

#### Transcriptomic data

Seven probes were associated in the bronchial brushing samples with submucosal eosinophils after adjustment for age and gender and correction for false discovery rate (figure 4). Five probes (1553569\_PM\_at, 211239\_PM\_s\_at, 207308\_PM\_at, 224321\_PM\_at and 237070\_PM\_at) mapped to genes: *COX-2* (cyclo-oxygenase-2), *ADAM-7* (disintegrin and metalloproteinase domain-containing protein 7), *SLCO1A2* (solute carrier organic anion transporter family member 1A2), *TMEFF2* (transmembrane protein with epidermal growth factor like and two follistatin like domains 2) and *TRPM-1* (transient receptor

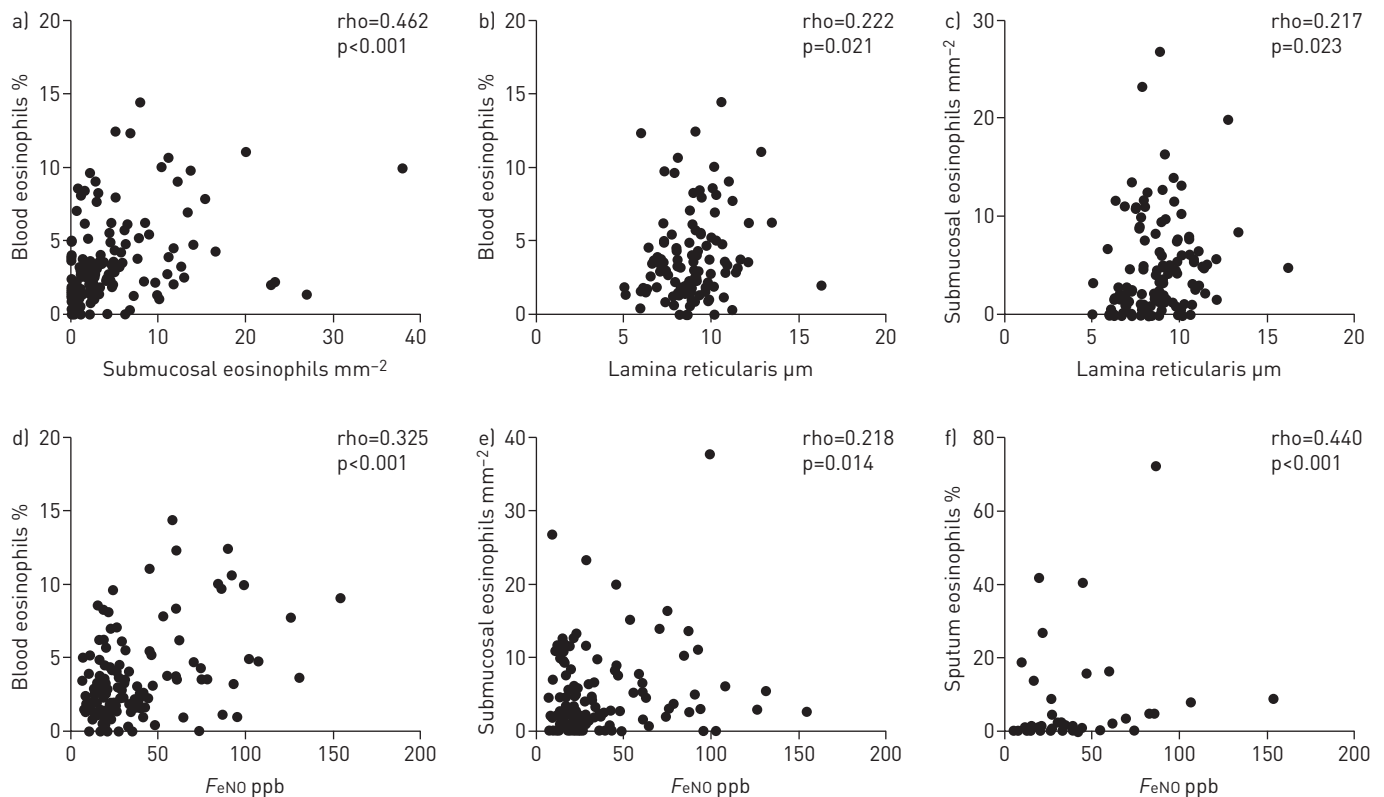


FIGURE 2 Immunopathology correlations. There was a positive relationship between a) blood eosinophils and submucosal eosinophils, and both b) blood eosinophils and c) submucosal eosinophils with the thickness of the lamina reticularis. Eosinophils in d) blood, e) submucosa and f) sputum correlated with exhaled nitric oxide fraction (FeNO).



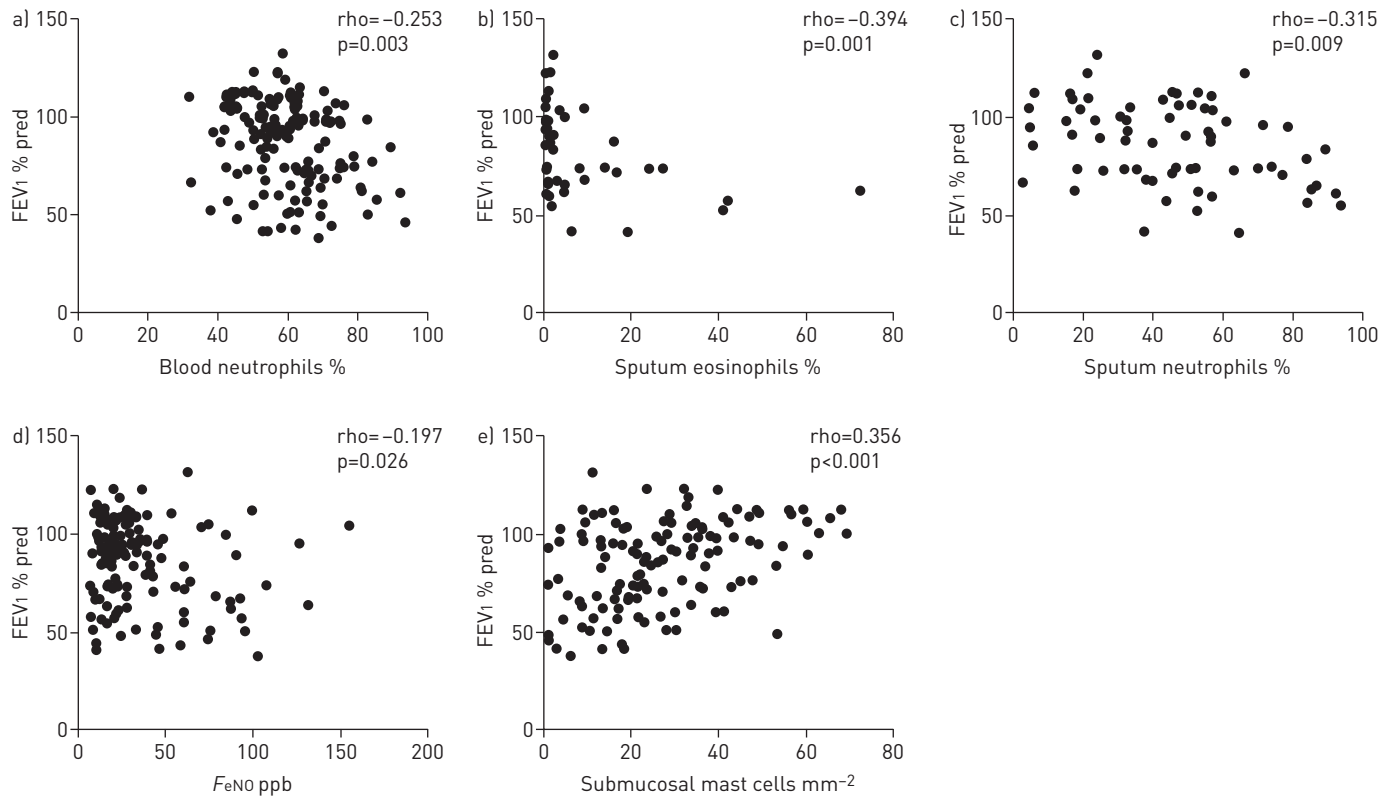


FIGURE 3 Immunopathology and clinical correlations. There was an inverse relationship between forced expiratory volume in 1 s (FEV1) % pred and a) blood neutrophils, b) sputum eosinophils, c) sputum neutrophils and d) exhaled nitric oxide fraction ( $FeNO$ ), and a positive relationship with e) submucosal mast cells.

potential cation channel subfamily M member 1). The remaining two were unmapped: 224372\_PM\_at and 224375\_PM\_at. There was no significant relationship with submucosal neutrophils.

After correction there were no significant correlations between gene expression, at the individual gene level, and either submucosal eosinophils or neutrophils in the bronchial biopsies.

### Stratification

The cut-off for grouping into low or high eosinophils within the submucosa, based on the U-BIOPRED HC group data (mean+2SD), was 12.63 eosinophils  $mm^{-2}$ . This resulted in 11 participants being allocated to the eosinophil-high group; the remaining (n=126) were in the eosinophil-low group. There were no differences in the clinical characteristics between the eosinophil-low and -high groups. The percentage of eosinophils in the blood and sputum, and the numbers of submucosal eosinophils, macrophages, CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> and CD25<sup>+</sup> T-lymphocytes were all significantly higher in the eosinophil-high group compared with the eosinophil-low group ( $p<0.05$ ) (table 3). Only five participants with asthma had submucosal neutrophil counts outside the mean+2SD range of the healthy controls. This was too few for statistical comparisons.

### Discussion

The participants in this bronchoscopy study were representative, with regard to clinical characteristics, and blood and sputum cells counts, of those in the main U-BIOPRED study [19]. Here, we added bronchial immunopathology to our initial study [19], and show that in both severe and mild-moderate asthma there are less mast cells within the bronchial submucosa than in healthy individuals. Also, in severe asthma smokers there are less CD4<sup>+</sup> lymphocytes in the bronchial submucosa compared with severe asthma nonsmokers. We observed relationships between the inflammatory profiles in the different airway compartments and with clinical measures of disease. Stratification by biopsy eosinophilia revealed a typical type 2 pattern of inflammation. Finally, we identified seven gene probes in the bronchial epithelial cells that were related to biopsy eosinophilia. These data indicate that those with stable severe asthma on adequate therapy do not have major changes in inflammatory cell infiltration within the bronchial mucosa of their large airways, suggesting that disease severity is mostly driven by other mechanisms, such as cellular activation status or inflammation elsewhere. There were 158 eligible participants in this

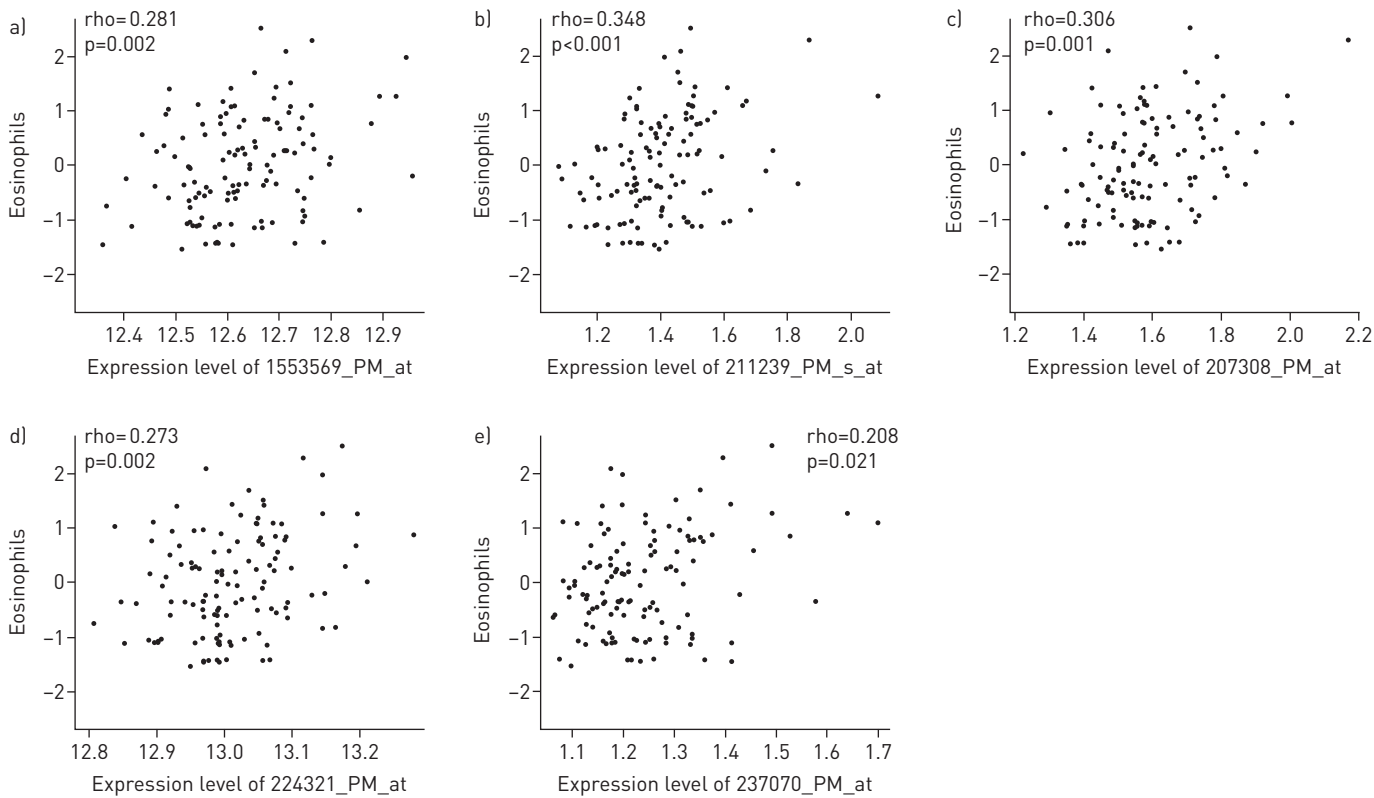


FIGURE 4 Transcriptomic data. The expression of five probes sets (log<sub>2</sub> normalised intensity) for a) *COX-2* (cyclo-oxygenase-2), b) *ADAM-7* (disintegrin and metalloproteinase domain-containing protein 7), c) *SLC01A2* (solute carrier organic anion transporter family member 1A2), d) *TMEFF2* (transmembrane protein with epidermal growth factor like and two follistatin like domains 2) and e) *TRPM-1* (transient receptor potential cation channel subfamily M member 1) associated with submucosal eosinophils (adjusted for age and sex) in the bronchial brushing samples.

bronchoscopy study and we had analysable biopsies from 137 (87%) of these. This success was due to the harmonisation of the SOPs across all centres, training in bronchoscopy and biopsy handling, and ongoing feedback to clinical centres about the quality of collected biopsies. To the best of our knowledge, this represents the largest cohort with such a comprehensive sample collection combined with “omics” measurements.

Several studies have reported a reduction in submucosal mast cells with inhaled and oral steroid therapy [24–26], and BALZAR *et al.* [27] in the SARP study report reduced numbers of tryptase-positive mast cells in airway biopsies in severe asthma. Thus, the reduction in mast cells we have observed is likely due to the effects of inhaled corticosteroid treatment that all of our asthma subjects were taking. There was a lack of any other asthma-related airway inflammatory signal at this proximal airway site, including significant

	Group		p-value
	Eosinophil-low <sup>#</sup>	Eosinophil-high <sup>¶</sup>	
<b>Subjects</b>	126	11	
<b>Blood eosinophils %</b>	2.72 [1.5–4.28]	4.76 [2.36–8.85]	0.025
<b>Sputum eosinophils %</b>	0.33 [0–2.03]	4.64 [1.87–25.43]	0.048
<b>Submucosal eosinophils mm<sup>−2</sup></b>	2.17 [0.59–5.07]	16.48 [13.82–23.11]	<0.001
<b>Submucosal macrophages mm<sup>−2</sup></b>	2.17 [0.95–4.20]	7.94 [4.83–11.61]	0.006
<b>Submucosal CD3<sup>+</sup> lymphocytes mm<sup>−2</sup></b>	33.52 [17.92–47.87]	77.17 [45.12–96.78]	0.001
<b>Submucosal CD4<sup>+</sup> lymphocytes mm<sup>−2</sup></b>	9.57 [4.41–17.78]	23.91 [10.74–30.45]	0.003
<b>Submucosal CD8<sup>+</sup> lymphocytes mm<sup>−2</sup></b>	15.38 [7.14–25.56]	37.42 [29.25–44.04]	<0.001
<b>Submucosal CD25<sup>+</sup> lymphocytes mm<sup>−2</sup></b>	0 [0–0]	0.79 [0–1.04]	<0.001

Data are presented as n or median [interquartile range], unless otherwise stated. <sup>#</sup>: ≤12.63 mm<sup>−2</sup>; <sup>¶</sup>: >12.63 mm<sup>−2</sup>.



airway eosinophilia, which is also reported in the SARP study [15]. This is also likely to be a reflection of the steroid treatment, as type 2 inflammation has been shown to be highly steroid sensitive [16]. In this study this is evidenced by the upregulation of gene expression of a common molecular marker of glucocorticosteroid response, FK506 binding protein 5 (*FKBP5*) [28], in the bronchial epithelial cell gene array analysis. The *FKBP5* epithelial gene signal was significantly increased in all of the three asthma groups compared with the HC group (see online supplementary material). Additionally the Medicines Adherence Response Scale scores recorded for these three asthma groups were in the range of 21–22, with the severe asthma groups recording higher scores, suggesting good adherence with steroid therapy.

We found no difference in the thickness of the lamina reticularis in severe asthma compared with healthy controls. There are some reports of this being a hallmark of asthma that relates to severity [7], but others like us do not observe an increase [5, 6]. There was, however, a correlation between markers of eosinophilic inflammation and lamina reticularis thickness suggestive of the link between these two processes. This is consistent with a previous report identifying that the lamina reticularis thickness was significantly higher in eosinophilic as compared with noneosinophilic severe asthma and that it was only in eosinophilic severe asthma that there were differences from that in healthy individuals [13].

To the best of our knowledge, there are no previous studies examining the association of smoking with airway inflammation in severe asthma. A reduction in CD4<sup>+</sup> lymphocytes, as observed by us, has been reported when comparing smoking and nonsmoking mild asthma with occupational asthma [29]. We also saw an increase in absolute numbers of neutrophils in the blood and a decrease in FeNO in the SAs/ex group compared with the SAn group. These effects are also reported in milder asthma [30–32]. Other inflammatory changes observed in response to smoking in milder asthma include a reduction in blood [30] and sputum eosinophils [30, 33], increases in sputum neutrophils [29], and increased submucosal CD8<sup>+</sup> lymphocytes and macrophages in bronchial biopsies [34]; however, we did not see these differences in our severe asthma subjects.

When attempting to stratify our data by eosinophilia, the majority of the participants had tissue eosinophil counts within our defined normal range (mean+2SD of healthy controls), consistent with effective glucocorticoid-related anti-inflammatory effects within their proximal airways or having a noninflamed bronchial mucosa. However, the 11 participants who were in the eosinophil-high group had higher percentages of eosinophils in the blood and sputum, greater numbers of submucosal macrophages, CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> and CD25<sup>+</sup> T-lymphocytes, and higher numbers of epithelial eosinophils than the eosinophil-low group. This is consistent with a type 2 inflammatory response. This needs to be interpreted cautiously due to the small numbers, but could represent a group that is either refractory to steroid therapy or noncompliant with medication. Two of the participants in this group were healthy controls. One of these individuals was atopic and had seasonal allergic rhinitis, and the other had nasal polyps, *i.e.* factors that can lead to inflammation within the lower airways [35].

One of the strengths of the U-BIOPRED bronchoscopy study was that it not only assessed proximal tissue events but also assessed inflammatory cell numbers in sputum and blood, as well as FeNO concentrations. In the SAn group there were increases in both sputum eosinophils and neutrophils compared with the HC group, and this SAn group eosinophilia was also greater than in the MMA group. Despite no changes in these cells in the bronchial mucosa there was a positive relationship between the number of eosinophils in the blood, but not sputum, and in the bronchial submucosa, epithelium and ASM across the groups. This could suggest that there is ongoing recruitment and trafficking of eosinophils into the airway lumen. These eosinophil changes were accompanied by elevated FeNO in the SAn group compared with the HC group, but not the MMA group. FeNO is reported to correlate with airway eosinophilia [36, 37] and we observed a positive relationship with submucosal eosinophils with FeNO when assessed across all groups.

We identified seven associations in the bronchial brushings that were linked to submucosal eosinophils. These included probes for *COX-2*, *ADAM-7*, *SLCO1A2*, *TMEFF2* and *TRPM-1*, and two unnamed genes. This was obtained by using a very stringent level of false discovery, which was required as this present study did not include an external validation group. The enzyme COX-2 is induced during inflammation and is involved in the synthesis of prostaglandins, converting arachidonic acid to prostaglandin H<sub>2</sub> [38]. Its expression, which is localised to the bronchial epithelium and submucosal inflammatory cells, is reported to be increased in expression in mild asthma and aspirin-sensitive asthma compared with healthy controls [39–41]. To the best of our knowledge, there are no reports of its expression levels in severe asthma. The relevance of the other genes is less clear, as there are no reports of these in asthma.

From this U-BIOPRED bronchoscopy study of 96 participants with asthma and 41 healthy controls, we conclude that in nonsmoking and smoking patients on currently recommended therapy, severe asthma exists despite suppressed endobronchial tissue inflammation within the proximal airways. This suggests that additional mechanisms in central airways or altered peripheral airway changes are contributing to asthma severity.

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