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The relationship between eosinophilia and airway remodelling in mild asthma

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Abstract

Background—Eosinophilia is a marker of corticosteroid responsiveness and risk of exacerbation in asthma; while it has been linked to submucosal matrix deposition, its relationship to other features of airway remodelling is less clear.

Objective—The aim of this study was to investigate the relationship between airway eosinophilia and airway remodelling.

Methods—Bronchial biopsies from subjects (n=20 in each group) with mild steroid-naïve asthma, with either low (0 – 0.45 mm⁻²) or high submucosal eosinophil (23.43 – 46.28 mm⁻²) counts and healthy controls were assessed for *in vivo* epithelial damage (using EGFR staining), mucin expression, airway smooth muscle (ASM) hypertrophy and inflammatory cells within ASM.

Results—The proportion of *in vivo* damaged epithelium was significantly greater (p=0.02) in the eosinophil-high (27.37%) than the eosinophil-low (4.14%) group. Mucin expression and goblet cell numbers were similar in the two eosinophil groups; however, MUC-2 expression was increased (p=0.002) in the eosinophil-high group compared to controls. The proportion of submucosa occupied by ASM was higher in both asthma groups (p=0.021 and p=0.046) compared to controls. In the ASM, eosinophil and T lymphocyte numbers were higher (p<0.05) in the eosinophil-high group than both the eosinophil-low group and the controls, whilst the numbers of mast cells were increased in the eosinophil-high group (p=0.01) compared to controls.

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CONFLICTS OF INTEREST

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Conclusion—Submucosal eosinophilia is a marker (and possibly a cause) of epithelial damage and is related to infiltration of airway smooth muscle with eosinophils and T lymphocytes but is unrelated to mucus metaplasia or smooth muscle hypertrophy.

Keywords

Asthma; eosinophil; remodelling; epithelium; goblet cell; smooth muscle; inflammation

INTRODUCTION

Airway eosinophilia and remodelling are hallmarks of asthma [1–4] but how they are related is not well established. It is increasingly recognised that asthma has several phenotypes [5,6], including eosinophilic and non-eosinophilic, which may reflect different underlying pathologies. The eosinophilic phenotype has been linked to steroid responsiveness, asthma exacerbations and severe or fatal asthma [7–15]. Recent analysis of pooled data of the sputum counts from several clinical trials reports almost equal incidence of eosinophilic and non-eosinophilic phenotypes in mild-moderate asthma [16]. The use of cluster analysis has also identified several further clinical [17] and mixed clinical/pathological phenotypes [5,18].

Eosinophils have the potential to drive airway remodelling [19]. Reduction of eosinophil numbers in the airway mucosa with anti-IL-5 monoclonal antibody treatment, Mepolizumab, [20–23], reduces asthma exacerbations [21–23] and also the expression of extra-cellular matrix proteins in parallel with attenuated expression of the pro-fibrotic tissue transforming growth factor (TGF)- β [20]. In further support of the concept, increased thickening of the *lamina reticularis* has been reported in both severe [7,9] and mild-moderate asthmatics [18] with airway eosinophilia when compared to asthmatics of similar severity but without eosinophilia. Moreover, Minshall *et al* [24] showed a correlation between TGF β expression, asthma severity and submucosal collagen deposition and, although their study did not show a direct relationship between eosinophil numbers and the extent of submucosal fibrosis, eosinophils were identified as the main source of TGF β and their numbers correlated with lung function.

In this study we have focused on identifying the relationship between eosinophilia and other features of airway remodelling, including epithelial damage, goblet cell hyperplasia and smooth muscle hypertrophy. First, we sought to elucidate the relationship between eosinophils and epithelial changes. Whilst post-mortem studies have shown evidence of epithelial damage in asthma [25,26], confirmation of this in biopsies from living asthmatics has been difficult [27–30] primarily because of difficulties in distinguishing *in vivo* acquired damage and artefactual damage during bronchoscopic biopsy [29]. In this study we used the expression of Epidermal Growth Factor receptor (EGFR) to distinguish between epithelium damaged *in vivo* from that damaged during bronchoscopic sampling and/or biopsy processing. Our second hypothesis was that eosinophilia is a determinant of goblet cell hyperplasia. Whilst some studies have shown increased mucus in the airways of patients with severe or mild asthma [2–4], other studies have not [28], possibly reflecting differences in asthma severity, treatment or the degree of accompanying inflammation, but none of the

studies to date have sought to link mucus hyperplasia and inflammation. Finally, we hypothesised that eosinophilic infiltration of airway smooth muscle (ASM) may be another hallmark of asthma and that this may be related to the mast cell and T lymphocyte infiltration previously reported to be increased in ASM in patients with asthma, particularly those with severe disease [31–33].

METHODS

Study design and subjects

For this current study atopic asthma subjects were randomly selected from a pool of 103 steroid naive subjects with mild-moderate asthma who had undergone full clinical evaluation and baseline bronchoscopy with collection of bronchial biopsies, as part of a previously published study [34]. For inclusion in the original study [34] the asthmatic subjects had to have a diagnosis of asthma for 6 months, with FEV₁ between 60–90% predicted, reversibility to β -agonists $\geq 12\%$ or a PC₂₀ to methacholine $< 8\text{mg/ml}$ if they did not demonstrate reversibility. They had to be atopic and non-smoker for at least one year, with a history of less than ten pack-years. For 6 weeks prior to bronchoscopy subjects were on their “standard” asthma treatment that included no other asthma medication than a short-acting b2 agonist on demand and free from respiratory tract infections, and they should not have been hospitalised for asthma within the past 12 months.

Biopsies were obtained and processed as previously described [34] and all samples were shipped for analysis at a single site. The biopsies were evaluated for eosinophil numbers by staining immunohistochemically (IHC) with the anti-eosinophil cationic antibody, EG2. As the eosinophil data were not normally distributed the subjects stratified into those with high submucosal eosinophil (eos-high, 39.04 eosinophils mm^{-2} submucosa) counts and those with low eosinophil (eos-low, zero eosinophils) counts using the upper and lower quartiles of the whole dataset (figure 1). Subjects were selected for analysis using a standard random selection method by a person independent from the study and blinded to the subject details. Additionally bronchial biopsies were collected from 20 healthy, non-atopic controls subjects with a PC₂₀ metacholine greater than 16 mg/ml. The subject characteristics are summarised in table 1. Subject groups were age matched. The mean FEV₁ % predicted did not differ between the eos-low (77% predicted) and eos-high (76% predicted) asthma groups but was significantly ($p < 0.001$) lower than in control subjects (98% predicted). The bronchial biopsies were further analysed by immunohistochemistry for epithelial damage, mucin expression, and hypertrophy of and inflammatory cell influx into the ASM.

The study was conducted in accordance with the principles of the Declaration of Helsinki. Approval was obtained from the local ethics committee at each clinical centre (see acknowledgements for approval numbers) and subjects gave written informed consent.

Assessment of epithelial Integrity

All biopsies containing epithelium were analysed to avoid selection bias. Sections were stained for epidermal growth factor receptor (EGFR) (rabbit anti-EGFR Cell Signalling, Hitchin, UK). This has previously been shown by Puddicombe *et al* [35] to enable

distinction of epithelium with a damaged morphology acquired *in vivo* - consequently staining positively for EGFR - from that damaged during biopsy and/or biopsy processing and, therefore, displaying negative immunoreactivity for EGFR. Using computer assisted image analysis (Zeiss KS400, Image Associates, Bicester, UK) the following epithelial lengths were measured: total epithelial length, intact epithelium (full height well orientated with a pseudostratified appearance) *in vivo* damaged epithelium (EGFR+) and artefactually damaged epithelium (EGFR-)(figure 2).

Goblet cell hyperplasia and epithelial mucin phenotype

All biopsies with intact epithelium in each of the subject groups were immunostained with monoclonal antibodies to MUC2 (BD Pharmingen, Oxford, UK), MUC4 (Zymed laboratories, Paisley, UK), MUC5AC (Chemicon, Southampton, UK) and MUC5B (Chemicon). The percentage of stained epithelium for each mucin was assessed by computerised image analysis and results expressed as area ratio, based on the red/green/blue colour composition of the DAB staining [35] within the intact epithelium. Additionally, sections were stained by the periodic acid Schiff (PAS) technique to identify all goblet cells. The numbers of PAS positive goblet cells were counted and reported as a percentage of the total number of epithelial cells.

Smooth muscle proportion and inflammatory cell infiltration

Sections from all biopsies containing smooth muscle were stained with alpha smooth muscle actin (Sigma, Poole, UK) to delineate the ASM and with antibodies to mast cells (AA1+, Abcam, Cambridge, UK), eosinophils (EG2+, Diagnostics Development, Upsala, Sweden), neutrophils (elastase+, Dako, Ely, UK) and T lymphocytes (CD3+, AbD Serotec, Kidlington, UK). A point counting grid was used on the sections stained for alpha smooth muscle actin in order to assess the muscle proportion, i.e. the volume fraction (proportion) [36] of ASM within the mucosa. The nucleated inflammatory cells within the smooth muscle were also counted and expressed relative to the area of ASM measured by computerised image analysis.

Statistical Analysis

Non-parametric statistics were used to analyse the results of Immunohistochemical analysis. The Kruskal-Wallis ANOVA test was initially used to test for differences between the 3 groups, with $p < 0.05$ considered statistically significant. If a significant difference was observed between the three groups, the Mann Whitney U test was used for further analyses.

RESULTS

The results of immunohistochemical analysis are presented in figures 3 to 5 and in table 1 in the online supplement.

Integrity of the epithelium

Assessment of epithelial integrity was made in a mean epithelial length of 13.44 mm in a mean of 4.59 biopsies per subject. The length of intact and *in vivo* damaged (EGFR+) epithelium differed between the 3 groups (Kruskal Wallis $p < 0.001$ and $p = 0.04$ respectively),

whilst the length of artefactually damaged (EGFR⁻) epithelium did not ($p=0.256$; data not shown). Further analysis showed that the length of intact epithelium with a pseudostratified appearance was significantly lower in the eos-high group (3.35%) compared to both the eos-low group (9.32%) ($p=0.01$) and the healthy control subjects (16.24%) ($p<0.001$) (Figure 3a). Also there was significantly more ($p=0.02$) *in vivo* damaged (EGFR⁺) epithelium in the eos-high group (27.37%) when compared to the eos-low group (4.14%) but not the healthy control subjects (figure 3b).

Goblet cell hyperplasia and epithelial mucin phenotype

Assessment of goblet cell hyperplasia and epithelial mucin phenotype was made in a mean intact epithelial length of 1.94 mm and in a mean of 2.75 biopsies per subject. No goblet cell hyperplasia was observed in the asthmatic groups (on-line supplement table) as the percentage of PAS positive cells did not differ between the groups. No staining for MUC4 was observed in any of the biopsies. There was a significant difference (Kruskal Wallis $p<0.001$) in the expression of MUC2 between the three groups but no differences in MUC5AC or MUC5B. Further analysis showed higher MUC2 expression in the eos-high asthmatics (0.70%) than the control subjects (0.0%) ($p=0.002$) but not the eos-low asthmatics (0%) (figure 4a). A representative photograph of MUC2 staining is shown in figure 4b.

Smooth muscle proportion and inflammatory cells

Representative photographs showing inflammatory cell influx into the ASM are shown in figure 5. A mean of 4.22 biopsies with a mean ASM area of 1.31mm^2 per subject were analysed. 79% of biopsies collected contained smooth muscle. There were significant differences between the three groups in the proportion of smooth muscle (Kruskal Wallis, $p=0.04$) and the numbers of inflammatory cells within this layer (Kruskal Wallis, eosinophils $p<0.001$, mast cells $p=0.023$ and T lymphocytes $p<0.001$). Further analysis indicated that the proportion of ASM occupying the submucosa was similar in the two asthmatic groups but was significantly higher in both the eos-high (33%) ($p=0.046$) and eos-low (33%) ($p=0.021$) groups when compared to healthy control subjects (26%) (figure 6a). The number of neutrophils within the ASM did not differ between the groups, while the numbers of eosinophils within the smooth muscle were significantly higher in the eos-high group ($0.54\text{ cells mm}^{-2}$) when compared both to the eos-low group (0) and the control subjects (0) ($p=0.004$, $p=0.002$) (figure 6b). The numbers of mast cells were also higher in the eos-high group ($12.88\text{ cells mm}^{-2}$) compared to the control subjects ($4.15\text{ cells mm}^{-2}$) ($p=0.008$) but not when compared to the eos-low group ($7.12\text{ cells mm}^{-2}$) (figure 6d). Finally, the numbers of T cells were higher in the eos-high group compared to both the eos-low group ($p=0.03$) and the controls ($p=0.03$) (figure 6c).

DISCUSSION

We have observed in steroid-naïve asthma, that high eosinophil numbers in the bronchial submucosa was associated with a greater degree of epithelial damage and increased numbers of eosinophils and T lymphocytes within the ASM. This extends previous studies in severe and mild-moderate asthma [6–10,15,16] by demonstrating that eosinophilic and non-

eosinophilic phenotypes can also be identified in mild to moderate asthma and are associated with some features of remodelling. Our study also shows that increased epithelial MUC2 expression and increased ASM mast cells are seen mainly in asthmatic subjects with high submucosal eosinophil counts, adding to the knowledge of mast cell myositis previously described as a hall-mark of asthma [31]. Finally, ASM hypertrophy was independent of concurrent eosinophilia. These important differences in airways pathology related to the extent of eosinophilic airway inflammation add to the notion that eosinophilia is an important marker of pathological as well as clinical phenotypes of asthma.

The issue of epithelial damage in asthma has been a matter of controversy [27–29]. In order to address the concerns that the observed damage may be an artefact of biopsy or tissue processing [29], in the current study we used EGFR immunostaining as a means of identifying true, *in vivo* acquired, damage (figure 2). The basis for this approach was the finding by Puddicombe *et al* [35] that in epithelial monolayer cultures damaged physically by scrape wounding and allowed to repair spontaneously the epithelial cells were negative for EGFR immediately after wounding but exhibited positive immunoreactivity after 3 hours that was still evident at 9 hours. In the same study, intense expression of EGFR was reported in the epithelium of bronchial biopsies from asthmatic patients. Here we observed that, when compared to healthy control subjects, epithelial integrity was reduced in asthmatics with high eosinophil counts, but not in those with low counts (figure 3a). This is in concordance with the study of Benayoun *et al* [30] which showed decreased epithelial integrity in subjects with intermittent asthma with eosinophil counts of 25.7/mm of length of epithelial basement membrane but not in asthmatics with <10 eosinophils/m² of mucosa. The percentage of *in vivo* damaged epithelium (EGFR+) correlated with eosinophilia (figure 3c) and was greater in eos-high than in eos-low asthmatics (figure 3b), in whom the damage was similar to the control group. This supports the argument that treatment that targets eosinophilic inflammation (e.g. corticosteroids) may also help in healing epithelial damage [30,37].

The association between increased eosinophil counts and decreased epithelial integrity suggests that eosinophils may damage the epithelium, probably through release of granule proteins, including major basic protein and eosinophil cationic protein, which were shown to induce epithelial cell damage *in vitro* [38–42]. On the other hand, eosinophils may be simply a biomarker of a more complex process involving several inflammatory cells and a susceptible epithelium.

In this study we have not seen evidence of goblet cell hyperplasia. This remodelling feature has been associated previously with severe asthma [43–45], but reports in milder asthma have been less clear. Similar to the current study, Lozewicz *et al* [28], who employed a point counting method, did not observe any difference in the percentage of epithelial cells that were goblet cells, whereas Ordonez *et al* [46] reported an increase in the number of goblet cells per unit of epithelial volume. There have been reports [18,46] of an increase in mucin volume (hypertrophy) in mild asthma, a measure that was not conducted in our study. The finding of a small but significant increase in MUC2 is, to our knowledge, novel. Previous studies have reported increased mRNA for MUC2 in the bronchial mucosa in asthma [87,46,47]; thus our study suggests that this is being translated into protein. MUC2 is

reported to be a minor component of airway mucus. However, its presence changes the physical properties of mucus and makes it more difficult to remove from the airways [48] and thus may contribute to mucus accumulation and plugging which are features of asthma [2–4].

The increase in the proportion of ASM observed in both asthma groups concurs with previous reports [30,49]. The mechanism by which this hypertrophy occurs is unclear. It may be due to increased smooth muscle cell size or cell numbers. Woodruff *et al* [49] have reported no change in cell size but an increase in cell numbers, whereas Benayoun *et al* [30] have reported an increase in cell size but no evidence of smooth cell proliferation which could lead to increases cell numbers. These studies have used different methodological approaches, which may explain the discrepancies in the data. In another study Stewart *et al* [50] suggested that hypertrophy is due to a reduction in smooth muscle cell apoptosis which leads to an increase in cell numbers. Further studies are needed to clarify the mechanisms leading to the increase in smooth muscle.

Finally, we have found mild eosinophilic and T lymphocytic inflammation in the ASM layer. Together with observations of elevated numbers of mast cell [31–33], this would suggest that ASM inflammation is an important feature of asthma. The complex inter-play between these inflammatory cells and the ASM is beyond the scope of this paper but is likely to include a range of chemoattractants and growth factors [51,52]. Neither eosinophils nor T cells were observed in the healthy controls or the eos-low group, but there was a significant infiltration compared to both of these groups in the eos-high group, which would suggest a spill-over of the classic Th2 type inflammation into the ASM. In contrast, mast cells, although raised in asthmatics with high eosinophil counts, were also present in significant numbers in healthy subjects, suggesting that these cells also play a role in maintaining a normal function for ASM, although the nature of this interaction remains unclear. Previously, Berry *et al* [7] have shown mast cell infiltration into the ASM to be a feature of both non-eosinophilic and eosinophilic asthma when subjects were stratified based on sputum eosinophilia. However, in their study the number of submucosal eosinophils was considerably higher (median 4.4mm^{-2}) than the numbers in our low-eos group (median 0mm^{-2}). This may explain the differences in findings between our study and that of Berry and colleagues.

In summary, we have demonstrated that the proportion of *in vivo* damaged epithelium is increased in patients with asthma when compared to normal control subjects and that it is related to submucosal eosinophilia. We have also found that some features of airway remodelling (i.e. mucin expression, ASM proportion and goblet cell numbers) are pronounced in asthma irrespective of eosinophilia while others (i.e. MUC-2 expression) are noted in association with eosinophil infiltration. The number of eosinophils and T lymphocytes that infiltrate the ASM layer is related to submucosal eosinophil counts in asthmatics who have high eosinophil counts. These findings indicate that submucosal eosinophilia is related to and, therefore, a biomarker of epithelial damage and infiltration of eosinophils and T lymphocytes into the ASM. Further studies are required to dissect the role of eosinophils (independently or via interaction with other inflammatory cells) in the pathogenesis of airway remodelling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ASM	airway smooth muscle
DAB	diamino benzidine
EGFR	epidermal growth factor receptor
FEV₁	forced expiratory volume in 1 second
IHC	immunohistochemistry
mRNA	messenger ribonucleic acid
PAS	periodic acid Schiff
TGFβ	transforming growth factor beta

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baseline eosinophil numbers

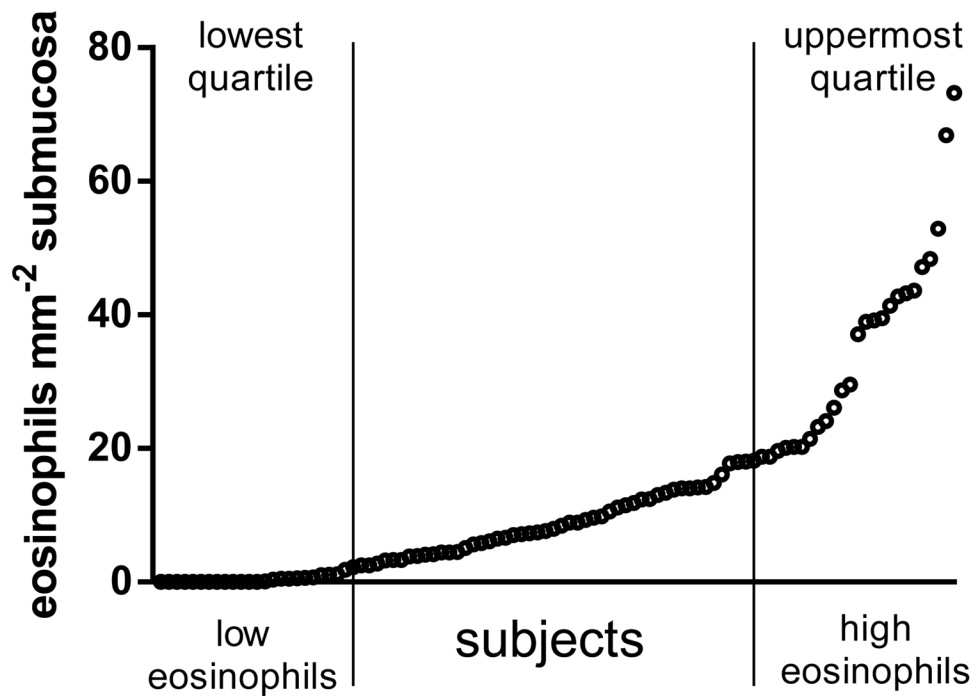
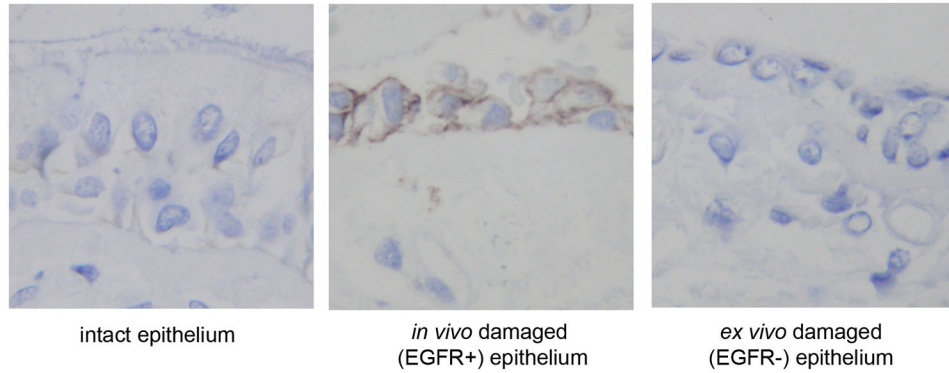


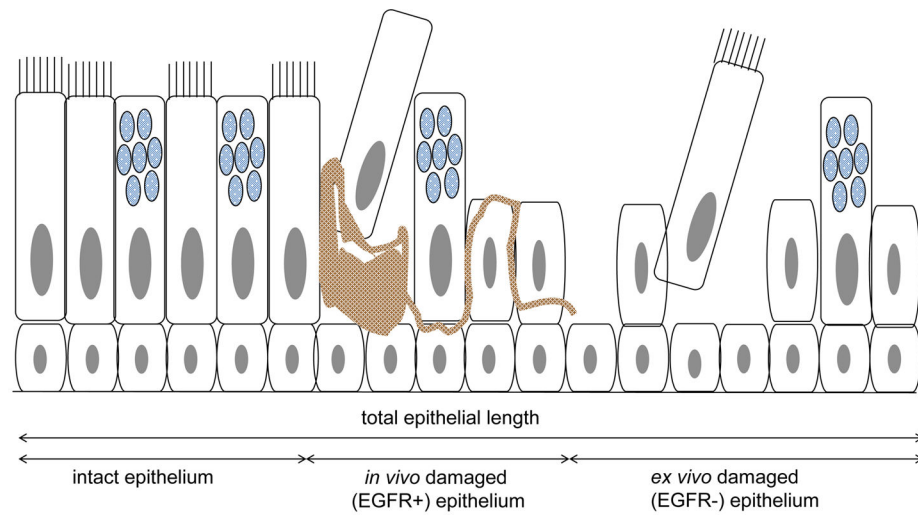
Figure 1. Eosinophil distribution in steroid naïve asthma

Subjects were stratified based on submucosal eosinophil numbers. The range for the whole cohort was zero to 73.21 eosinophils mm⁻². 20 subjects were randomly selected from the lowest quartile (0–2.21 eosinophils mm⁻²) (low eosinophils) and 20 from the uppermost quartile (18.41–73.21 eosinophils mm⁻²) (high eosinophils).

a: Photographic representation of epithelial phenotypes



b: Diagrammatic representation of measurements made

**Figure 2. Assessment of epithelial integrity**

Staining for EGFR was used to distinguish epithelium that had sustained damage *in vivo* (EGFR+)(a: middle plate) from that that had been damaged during bronchoscopy or processing (EGFR-)(a: right hand plate). Intact undamaged epithelium was EGFR negative (a: left hand plate). The total epithelial length and the lengths of intact, *in vivo* and *ex vivo* damaged epithelium were measured using computer assisted image analysis (b).

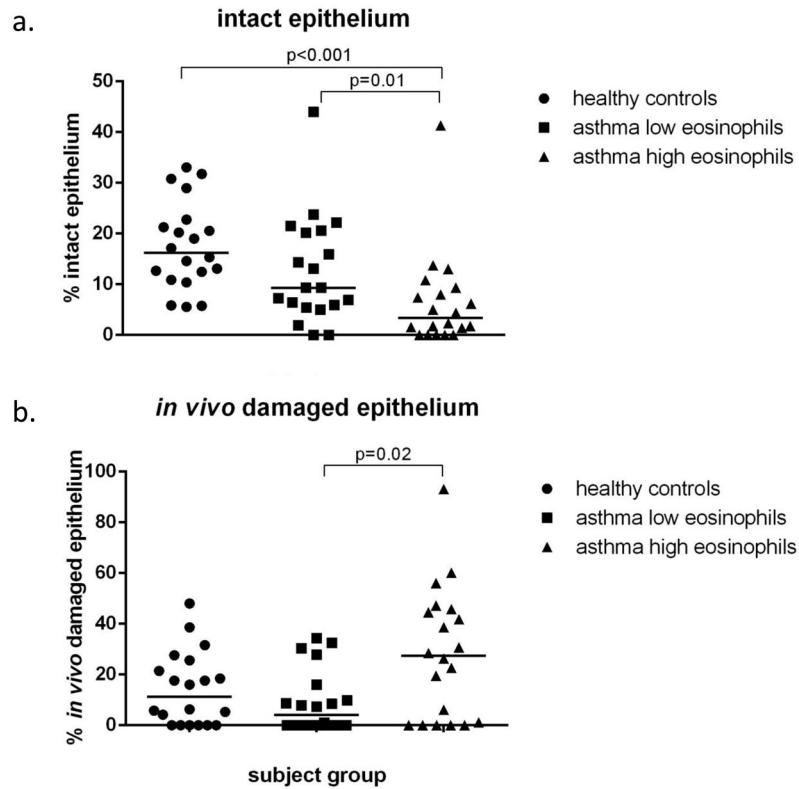


Figure 3. Integrity of the epithelium

The percentage of intact epithelium in the high eosinophil group is decreased compared to both healthy controls and asthmatics with low eosinophil counts (a). The *in vivo* damaged epithelium is increased in the eosinophil high group only in comparison to the eosinophil low group (b).

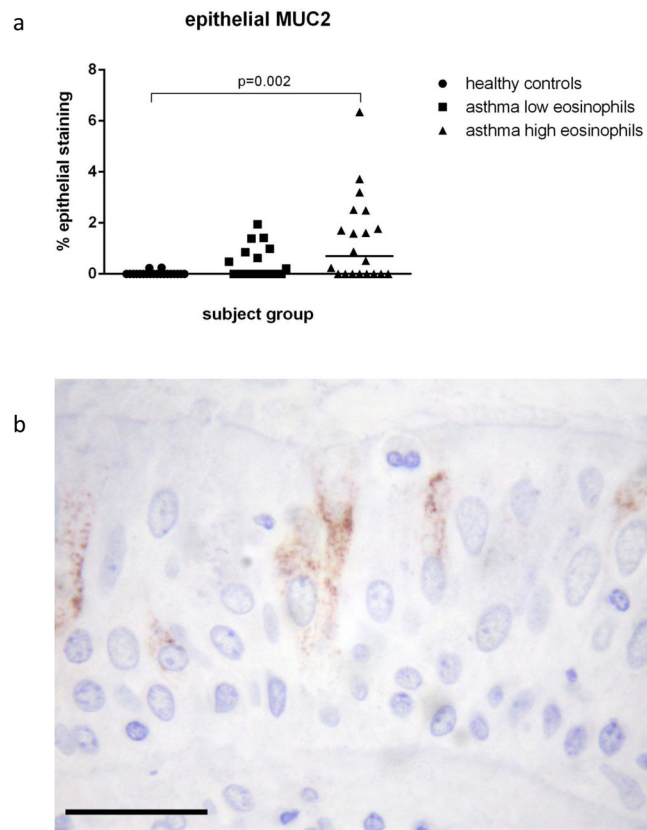


Figure 4. Epithelial mucin phenotype

Epithelial expression of MUC2 is higher in asthmatics with high eosinophil counts compared to healthy controls but not those with low eosinophil counts (a).

Immunohistochemical staining (brown) for MUC2 can be seen within the goblet cells. Scale bar is 50 μ m.

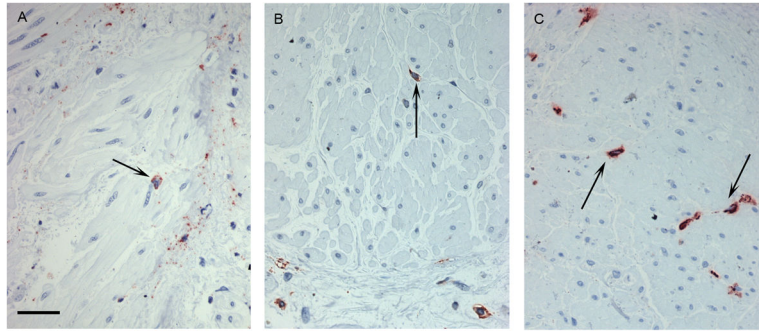
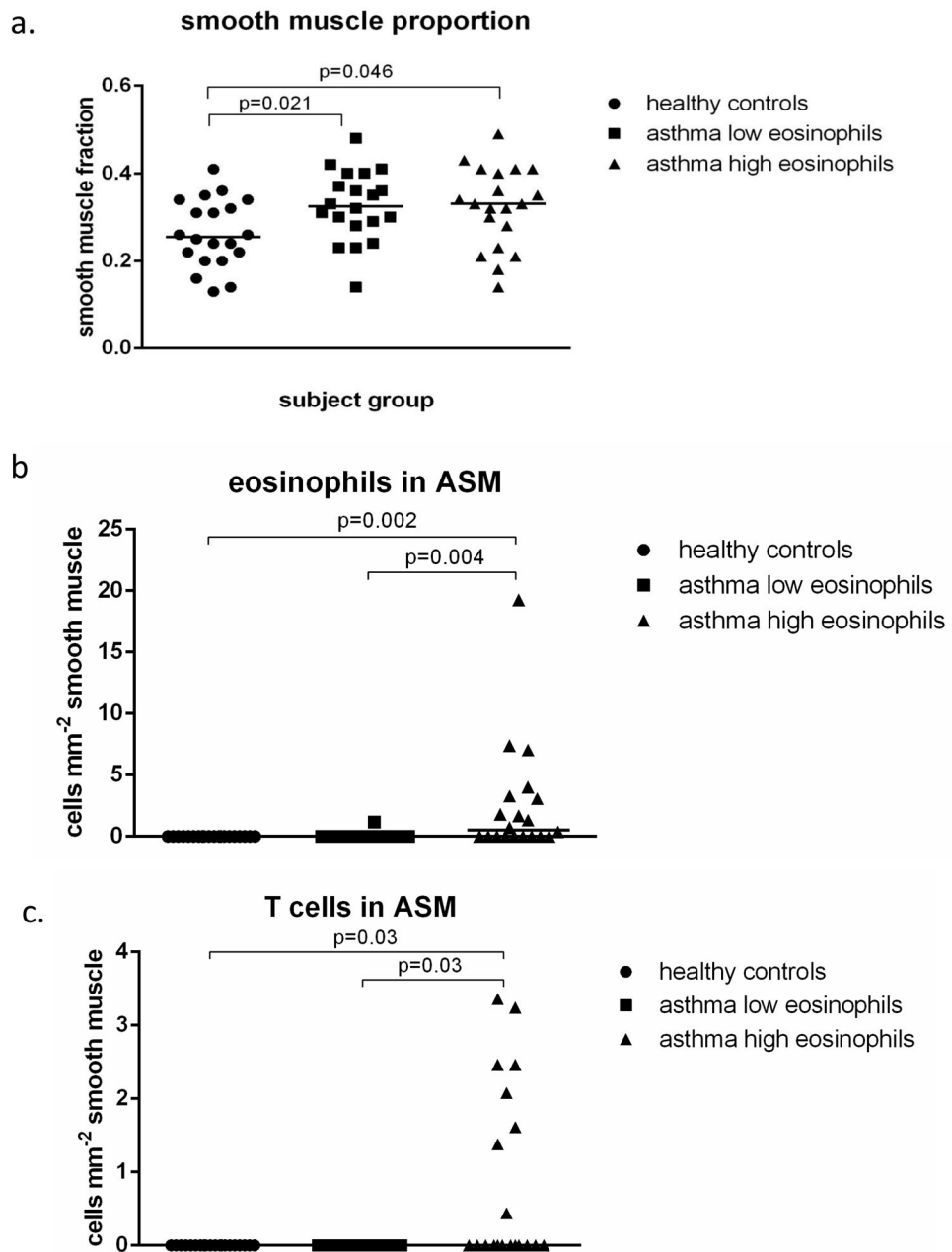


Figure 5. Influx of inflammatory cells into the airway smooth muscle
Immunohistochemical staining showing the influx of eosinophils (a), T cells (b) and mast cells (c) into the smooth muscle. Inflammatory cells are stained red and those within the ASM bundle were counted (↑). Scale bar is 50 μ m.



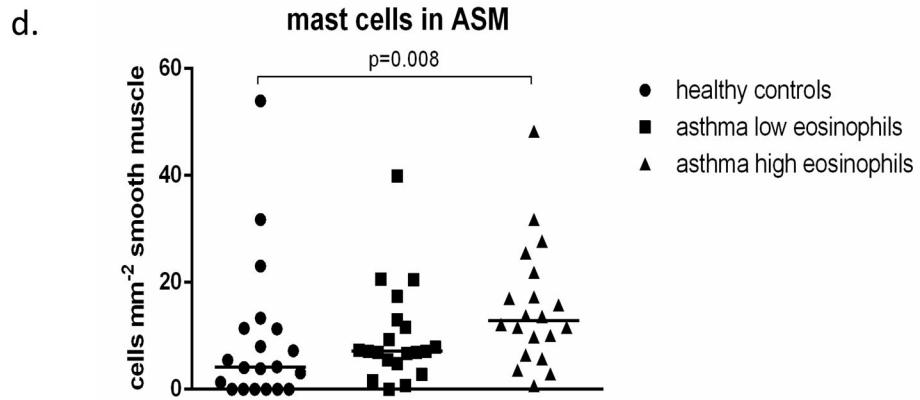


Figure 6. Smooth muscle proportion and inflammatory cell influx

The proportion of airway smooth muscle (ASM)(a) is increased in asthmatics with low and high eosinophil counts compared to healthy controls. Positive immunostained nucleated cells were counted in the ASM. More eosinophils (b) and T cells (c) are observed in the ASM of the asthmatics with high eosinophils counts compared to those with low counts and to the healthy controls. Mast cell numbers (d) are increased in subjects with high eosinophil counts compared to healthy controls but not low eosinophil counts.

Table 1

Subject Characteristics

Characteristic	Healthy controls	Low Eosinophils	High Eosinophils
Number of subjects	20	20	20
Age (years)	27.8 (19–43)	29.2 (20–55)	30.5 (18–57)
Gender: F/M	15/5	14/6	10/10
Duration of asthma: >=6 months to <1 year	not applicable	0	1
>=1 year to <5 years		3	4
>=5 years to <10 years		5	3
>=10 years to <15 years		3	4
>=15 years		9	8
Tobacco use: Former/never smoker	0/20	6/14	6/14
FEV ₁ (% predicted)	97.9 (81.7–119.0)	77.1 (61.9–87.7)	75.7 (60.0–88.9)
Reversibility (FEV ₁ % change)	not applicable	15.5 (0.6–42.3)	15.7 (7.4–29.9)
PC20 methacholine (mg/ml)	>16	1.2 (0.5–2.4)(n=2)	4.1 (1.2–7.0)(n=5)
Submucosal eosinophils (cells mm ⁻²)	3.58 (0–6.20)	0 (0–0.45)	39.04 (23.43–46.28)

Clinical data are means (ranges), cell data are medians and interquartile ranges