

ORIGINAL ARTICLE

Inflammatory biomarkers in airways of patients with severe asthma compared with non-severe asthma

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Summary

Background About 5–10% of patients with asthma suffer from poorly-controlled disease despite corticosteroid (CS) therapy.

Objective We determined whether there were any differences in inflammatory biomarkers between severe and non-severe asthma patients.

Methods Nineteen severe and 20 non-severe asthma patients were recruited and underwent collection of induced sputum, bronchoalveolar lavage (BAL) fluid and bronchial biopsies.

Results Biopsy results showed no differences in eosinophils (major basic protein positive), neutrophils, macrophages, T cells and mast cells in the bronchial submucosa. However, subbasement membrane (SBM) thickness and smooth muscle area were increased in the biopsies. No significant differences were observed in the induced sputum inflammatory cells. In BAL fluid, there was a significant increase in neutrophils but a significant decrease in macrophages. Eosinophil counts were non-significantly increased threefold in both sputum and BAL in severe asthma. Increased levels of IL-8 and IL-13 were found in sputum supernatants of severe asthma patients. There was a significant inverse correlation between post-bronchodilator forced expiratory volume in 1 s and provocative concentration of methacholine causing a 20% fall in FEV₁ with SBM thickness.

Conclusion Differences in inflammatory cells were observed mainly in terms of increased neutrophils and reduction in macrophage numbers in BAL fluid with a trend towards increased eosinophils in severe asthma compared with non-severe asthma. However, the most notable features are the increase in features of airway wall remodelling of SBM thickness and smooth muscle area.

Keywords airway smooth muscle, bronchial biopsies, eosinophils, severe asthma, subbasement membrane thickness

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Introduction

Patients with asthma are usually well controlled with inhaled corticosteroids (CS) and long-acting β_2 -agonists but a minority of patients continue to have uncontrolled asthma in spite of these treatments [1]. These patients with severe asthma suffer greater morbidity, face a higher risk of asthma death and consume a greater proportion of health resources than other asthmatic patients. Patients with severe asthma may also present with more severe symptoms, greater lung function impairment, or experience more frequent exacerbations [2]. The pattern of disease may differ between patients. Some severe asthma patients have repeated exacerbations but minimal

interval symptoms; others rarely exacerbate but have persistent airway obstruction. Therefore, a definition of severe asthma must encompass many aspects of severity in order to capture severe asthmatics with different clinical phenotypes. The American Thoracic Society's (ATS) definition requires the presence of at least two of seven different minor criteria, despite the use of high-dose inhaled CSs or the long-term use of oral steroids [3]. A European Respiratory Society workshop has proposed a definition for severe asthma, which is equally broad [4].

One of the hypotheses about severe asthma is that patients may present with an enhanced inflammatory response, or that the characteristic eosinophilic

inflammation in the airways of asthma is replaced by other types of cellular inflammation. Thus, there is some evidence that there is a subgroup of asthma patients with predominantly neutrophilic response measured in biopsy samples or in sputum samples and that the presence of neutrophils may be an indication of severe asthma [5, 6]. One of the difficulties with performing studies for inflammatory biomarkers in asthma is the influence of concurrent therapies taken by the patient, particularly CSs. Therefore in the analysis of data from severe asthma, part of the confounding effect of treatment could be offset by analysing data from non-severe asthma patients taking lesser amounts of medications. The persistence of any inflammatory response in the airways of patients with severe asthma may reflect the degree of CS insensitivity present in these patients [7, 8].

In the current study, we examined various inflammatory biomarkers collected from different compartments of the airways and lungs including sputum from the large airways, bronchoalveolar lavage fluid (BAL) from the proximal and distal airways and mucosal biopsies from the proximal airways from patients with severe asthma as defined by ATS criteria. We compared these findings with those from patients with non-severe asthma.

Methods

Patients

Asthma was diagnosed by a physician but had either forced expiratory volume in 1 s (FEV_1) reversibility $\geq 12\%$ or methacholine provocative concentration of methacholine causing a 20% fall in FEV_1 (PC_{20}) < 8 mg/mL. Current and ex-smokers of > 5 pack-years were excluded. Severe asthmatics ($n = 19$) were defined according to guidelines developed by the Severe Asthma Research Program based on ATS criteria [3]. They had either one or two major criteria for CS usage, and had three or more minor criteria with 13 having five or more. Patients who did not meet the criteria for severe asthma were classified as *non-severe asthmatics* ($n = 20$), and used ≤ 1000 μ g of inhaled beclomethasone dipropionate-equivalent/day. All participants gave informed consent to a protocol approved by the Ethics Committee of the Royal Brompton & Harefield NHS Trust/National Heart & Lung Institute.

Exhaled nitric oxide

Exhaled nitric oxide (eNO) levels were measured using an online chemiluminescence analyser (NIOX, Aerocrine, Stockholm, Sweden) at a constant expiratory flow of 0.05 L/s.

Fiberoptic bronchoscopy

The entry criteria for performing bronchoscopies in patients with severe asthma comprised of (i) an $FEV_1 \geq 45\%$ predicted before and $FEV_1 \geq 55\%$ predicted following bronchodilators, (ii) less than six asthma exacerbations in the previous 6 months to bronchoscopy, (iii) no asthma hospitalization within 6 months; (iv) no endotracheal intubation for asthma within 1 year; and (v) regular daily oral dose of < 20 mg of prednisolone.

Fibreoptic bronchoscopy was performed using topical anaesthesia with lidocaine to the upper and lower airways and with intravenous of midazolam (3–6 mg) and alfentanil (125 μ g). Warmed 0.9% NaCl solution was instilled (50 mL $\times$ 4) in right middle lobe and BAL fluid was recovered by gentle suction. Washed BAL cells were suspended in culture media (RPMI with 0.5% fetal calf serum, antibiotics and L-glutamine) and counted on a hemocytometer. Cytospins were stained with Diff Quick stain (Harleco, Gibbstown, NJ, USA) for differential cell counts.

Bronchial biopsies

Bronchial biopsies were taken from segmental and sub-segmental airways of the right lower lobe and were embedded in optimal cutting temperature medium and then snap-frozen in pre-cooled isopentane in liquid nitrogen and stored at -70°C . Cryostat sections (6 μ m) were obtained from biopsies, placed on poly-L-lysine-coated microscope slides and stained with haematoxylin and eosin [for measurement of subbasement membrane (SBM) thickness].

Sections fixed in acetone were washed, immunostained for eosinophils and neutrophils in bronchial mucosa using immunoperoxidase 3% in methanol for 30 min (Vectastain kit PK4002, Vector, Peterborough, UK) and incubated with mouse monoclonal antibodies to major basic protein (MBP; dilution 1 : 100; cat. no. MON6008-1, Monosan, the Netherlands), or to neutrophil elastase (NE; dilution 1 : 50; DAKO Cytomation, cat. no. M0756, DAKO, Denmark), or to anti-CD3 antibody (Clone PC3/188A, cat. no. M7193; dilution 1 : 10; DAKO, Ely, Cambridgeshire, UK), or to anti-CD68 (macrophages; Clone EBM11; cat. no. M0718; dilution 1 : 50, or to anti-tryptase (mast cells; Clone AA1; cat. no. M0752, Abcam; dilution 1 : 100) for 1 h at room temperature. Sections were washed in PBS and incubated 45 min with biotinylated mouse IgG secondary antibody, made up in PBS containing horse serum. 3,3'-diaminobenzidine (Sigma D5905, Sigma-Aldrich Company Ltd., Gillingham, UK) was used for brown staining. Sections were finally counterstained in haematoxylin (20% in tap water) and dehydrated through alcohols to xylene.

Tissue quantification

Eosinophils, neutrophils, macrophages, T cells and mast cells were counted in the bronchial submucosa (lamina propria) in a zone excluding submucosal gland and smooth muscle. All available fields were examined, and the results were expressed as the total number of cells per square millimeter of submucosa. SBM thickness (μm) was quantified by measuring total length of basement membrane present in the biopsy that was overlaid by at least a basal epithelial cell layer. The thickness was computed from the area and the length [9]. The area of the bronchial submucosa occupied by airway smooth muscle was determined as the percentage of smooth muscle area over the total submucosal area. All parameters were examined in sections from two to four different biopsies and the mean of these measurements recorded.

Sputum induction and processing

All subjects received inhaled salbutamol (400 μg) but only subjects with an $\text{FEV}_1 > 60\%$ of predicted were allowed to proceed to sputum induction. The subjects inhaled nebulized 3% NaCl solution from an ultrasonic nebuliser. At 4, 8 and 12 min, the subjects were asked to blow their nose and rinse their mouth with sterile water before coughing up sputum into a sterile container. Subjects received β -adrenergic bronchodilators if they developed bronchospasm.

A small proportion of the sputum was transferred to microcentrifuge tubes, diluted to 50% with PBS, vortexed vigorously and centrifuged at 14 000 g for 10 min at 4 °C. This supernatant without DTT was stored at –80 °C. The remainder of the whole sputum was transferred to a 50 mL centrifuge tube and an equal volume of dithiotreitol (DTT, 0.05%, Sputolysin, Calbiochem, Gibbstown, NJ, USA) was added. This mixture was aspirated several times with a sterile pipette and placed in a shaking water bath at 37 °C for 15 min. A total cell count was then performed using Trypan blue. Cytospins were made for differential cell counts from cell suspensions (5×10^5 cells/mL).

Assay of interleukin-8 and interleukin-13

The sputum supernatants and the BALs were assayed for IL-8 and IL-13 levels by ELISA (R&D, Abingdon, UK) following the manufacturers' instructions, with a lower detection limit of 31.25 and 93.74 pg/mL, respectively. Total protein concentration in the assay was determined by Bradford Assay (reagents from Bio-Rad, Hemel Hempstead, UK).

Data analysis

Data are presented as means $\pm$ SEM. The Mann-Whitney test was used to analyse the differences in the numbers of inflammatory cells, ASM area and SBM thickness between

the two groups. A P -value of <0.05 was accepted as significant.

Results

A comparison of the baseline data of patients with severe and non-severe asthma is presented in Table 1. Regarding the major and minor criteria of the patients defined according to the ATS definition that we used [3], 50% of severe asthmatics had both major criteria and all severe patients had at least three minor criteria or more: 13% had three, 25% had four, 31% had five and 31% had six minor criteria. In the non-severe asthma patients, only one subject had one major criterion, while 41% of the non-severe asthma patients had no minor criteria, 47% had one and 12% had two minor criteria. In this group, half were taking inhaled CSs while the rest did not.

Severe asthmatics had more severe airflow obstruction ($P < 0.01$) and bronchial hyperresponsiveness ($P < 0.05$). The fiberoptic bronchoscopy procedure was generally well tolerated by all patients. In two patients with severe asthma, there was a significant worsening of asthma that needed treatment with intravenous hydrocortisone and a 48-hour stay in hospital. Both patients recovered well.

Bronchial biopsies

An example of the immunohistochemical detection of various inflammatory cells in bronchial biopsy specimens is shown in Fig. 1. In total, biopsies from 17 severe and 16 severe patients were analysed in this way, but for each staining particularly in severe asthma patients there were lesser numbers that were analysable in terms of inflammatory cell profile, depending on the adequacy of the biopsy or staining properties.

There were no significant differences between the MBP^+ eosinophils, NE^+ neutrophils, CD3^+ T cells and tryptase-positive mast cells between the two groups of asthma patients (Fig. 2).

An example of SBM thickness and airway smooth muscle present in biopsy from a severe asthma patient is shown in Fig. 3. There was a significant increase in SBM thickness ($P < 0.02$) and the smooth muscle area ($P = 0.01$) in the biopsies from severe asthma patients compared with the non-severe asthma patients (Table 2; Fig. 3). There was a significant negative correlation between PC_{20} and the thickness of the SBM ($r = -0.45$; $P = 0.02$), but not with smooth muscle area. Post-bronchodilator FEV_1 also correlated inversely with SBM thickness ($r = -0.44$; $P = 0.01$) but not with smooth muscle area (Fig. 4).

Bronchoalveolar lavage

BAL total cell counts was not different between the two groups, but there was a significant increase in neutrophil

counts and a significant decrease in macrophage counts in the severe asthma group (Table 2; Fig. 5). Eosinophil counts

Table 1. Patient characteristics

	Non-severe	Severe	<i>P</i>
<i>N</i>	20	19	
Gender (male:female)	12/8	6/13	NS
Age (years)	40.0±2.4	41.4±2.6	NS
Atopic* (<i>n</i>)	19	17	NS
Oral prednisolone (<i>n</i>)	0	10	<i>P</i> ≤0.001
IgE (IU/L)	340.6±67.5	207.5±59.2	NS
ICS (BDP equivalent mcg)	526±167	2530±292	<i>P</i> ≤0.001
eNO	31.9±4.0	41.5±8.6	NS
Pre-bronchodilator FEV ₁	84±2.0	59±4.6	<i>P</i> ≤0.001
Pre-bronchodilator FVC	94±3.5	73±4.9	<i>P</i> ≤0.01
FEV ₁ /FVC ratio (%)	75±2.1	66±2.4	<i>P</i> =0.02
Log PC ₂₀	0.19±0.14	-0.48±0.17	<i>P</i> ≤0.01
β-agonist reversibility [†] (%)	9.5±1.8	20.9±3.9	<i>P</i> ≤0.01

n, number of subjects; BAL, bronchoalveolar lavage; ICS, inhaled corticosteroids; BDP, beclomethasone dipropionate; mcg, micrograms; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; PC₂₀, provocative concentration of methacholine causing a 20% fall in FEV₁; eNO, exhaled nitric oxide levels in parts per billion (ppb); NS, not significant.

*Atopy defined as positive skin prick tests to one or more common aeroallergens.

[†]Measured as percent increase in FEV₁ after 400 µg salbutamol.

were non-significantly elevated. There was a negative correlation between BAL eosinophil (%) and log PC₂₀ ($r=-0.42$; $P=0.02$), and between BAL eosinophils (%) and eNO ($r=0.55$; $P<0.02$) in the severe asthma group.

Induced sputum cells

Induced sputum was not successful in two and eight of the non-severe and severe asthma patients, respectively. The total cell count recovery was non-significantly lower in the severe asthma patients, and there were no significant differences in the percentage of macrophages, lymphocytes, neutrophils and eosinophils (Fig. 6). The eosinophil count was approximately threefold higher in the severe asthmatic compared with the non-severe asthma patients but was not significantly different; this trend was similar for the eosinophils recovered in BAL fluid. Indeed, there was a significant correlation between the percentage of eosinophils in BAL and that in sputum for the severe asthma group ($r=0.57$; $P<0.002$).

Sputum interleukin-8 and interleukin-13 levels

Sputum levels of IL-8 and of IL-13 were significantly increased in severe asthma patients compared with non-severe asthma patients with levels being 45.8±17.7 vs. 22.4±4.6 ($P<0.01$) and 156.8±58.6 vs. 87.6±21.0 pg/µg protein ($P<0.02$), respectively (Fig. 7).

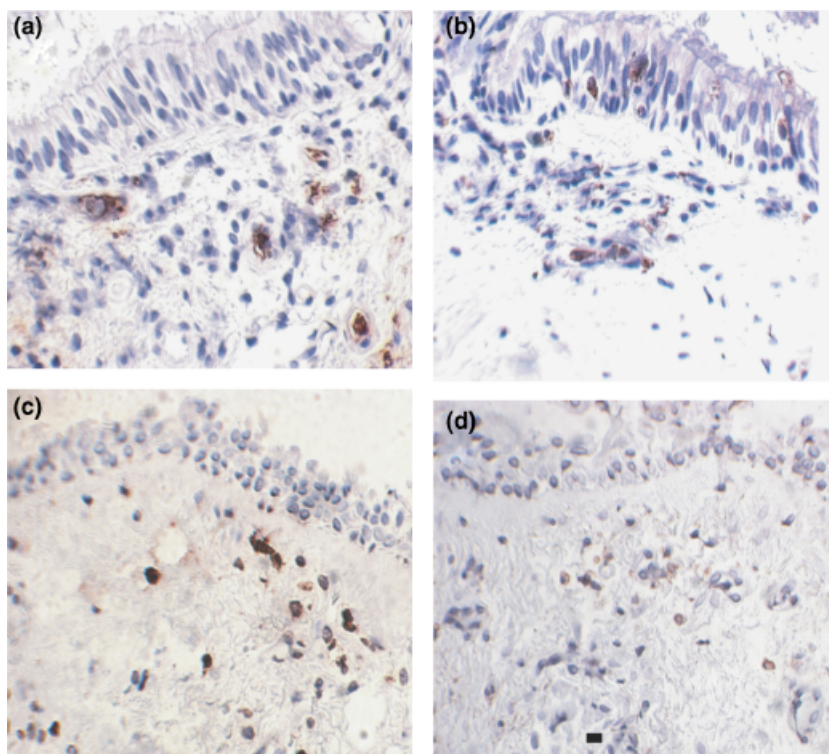


Fig. 1. Examples of bronchial biopsy sections from patients with severe asthma showing MBP⁺ eosinophils (a), CD68⁺ macrophages (b), neutrophil elastase-positive neutrophils (c) and CD3⁺ T cells (d).

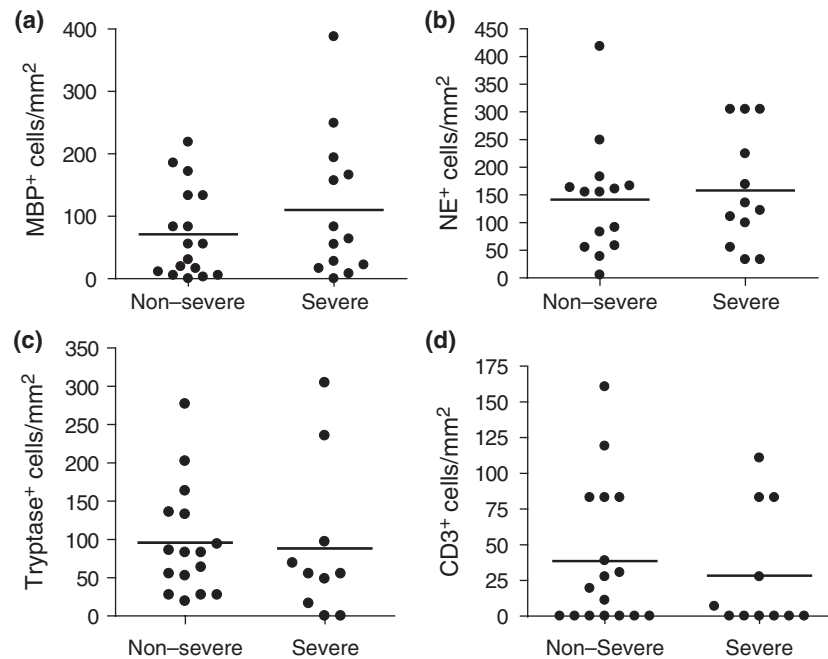


Fig. 2. Individual counts of eosinophil (MBP⁺, a), neutrophil (neutrophil elastase, NE⁺, b), mast cells (tryptase-positive, c) and lymphocytes (CD3⁺, d) from bronchial biopsies of non-severe and severe asthma patients. Horizontal bar indicates median values.

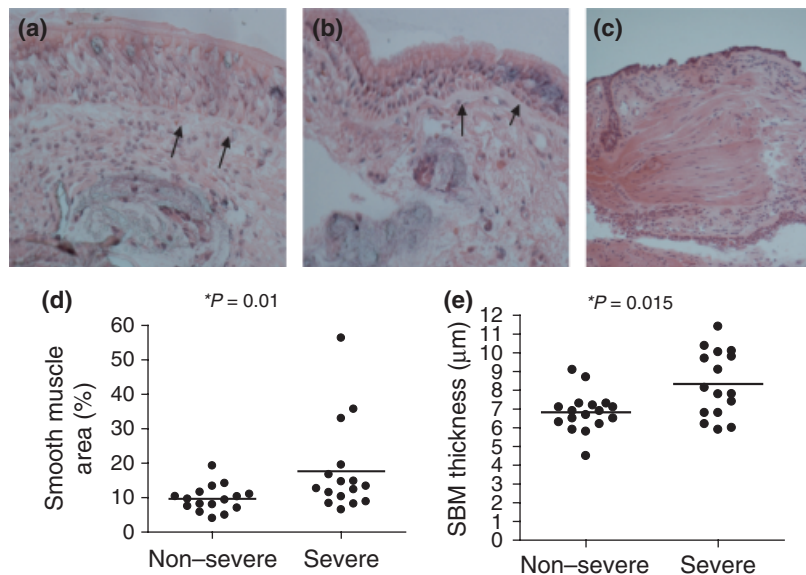


Fig. 3. Photomicrograph of bronchial biopsy section stained with haematoxylin and eosin showing subbasement membrane (SBM) thickness (arrows) in a non-severe asthma patient (a), and from a severe asthma patient (b). An example of airway smooth muscle content in a biopsy from a severe asthma patient is shown (c). Individual measurements of smooth muscle area (d), and SBM thickness (e) from bronchial biopsies of non-severe and severe asthma patients. Horizontal bar indicates median values.

Discussion

In this study of patients with severe asthma compared with patients with non-severe asthma, we have shown that there are very little detectable differences in the cellular inflammatory infiltrate in bronchial submucosa and in induced sputum; however, the BAL fluid composi-

tion showed an excess of neutrophils although the eosinophil counts as in sputum and biopsies showed a trend towards an increase. However, the most significant findings were those related to tissue remodelling, that is the increase in SBM thickening, evidence of increased sub-epithelial fibrosis and the increased airway smooth muscle area, evidence of an increased airway smooth muscle mass

in severe asthma compared with non-severe asthma. Our data would therefore indicate that the differences with severe asthma is an excessive amount of subepithelial fibrosis and airway smooth muscle mass, that has been previously reported in severe asthma [10–12]. Furthermore, the close relationship between bronchial hyperresponsiveness as measured by PC₂₀ and the degree of

subepithelial fibrosis that has also been previously reported also support the importance of this remodelling feature in severe asthma, because the severe asthma patients were differentiated from non-severe asthma by a greater degree of bronchial hyperresponsiveness. No relationship was found between baseline spirometric measurements and PC₂₀.

Our results must be interpreted in the light of the fact that all the patients in the severe asthma group were on high-dose inhaled CSs and that half of them were also taking oral CS therapy, while the non-severe asthma group were on no CS therapy or on lower doses of inhaled CS therapy, without oral CS therapy. The results of inflammatory cell numbers may be difficult to interpret between the two groups in the presence of unequal doses of inhaled and/or oral CS. The influence of high-dose CS therapy on airway inflammatory responses in the severe asthma patients is unclear. Studies of ICS therapy in non-severe asthma indicates that this therapy reduces eosinophil inflammation with a reduction in mucosal mast cells and T cells [13, 14]. If steroid responsiveness were unaffected in severe asthma, then one would expect to find lesser degrees of inflammation in severe asthma; however, no significant differences in eosinophils, mast cells and T cells were observed between the two groups in the various compartments measured with an equally wide distribution of inflammatory cells in both groups. By contrast, compared with non-asthmatic normal volunteers on no treatment, the degree of inflammatory cells in the submucosa is lower [15]. One could argue that this may be a reflection of relative CS insensitivity in the severe asthma patients, as has been shown recently [7, 8]. The only significant difference was found in terms of the BAL neutrophil count, which may be a direct effect of high doses of CS therapy [16]. However, neutrophil counts in the submucosa or neutrophil sputum counts were not significantly different between the groups. While there has been a neutrophilic group of patients with severe asthma that has been described characterized by sole increase in neutrophils in the biopsy submucosa [6], we did not find

Table 2. Endobronchial biopsies, BAL and sputum results

	Non-severe	Severe	P
<i>Endobronchial biopsies (n)</i>	17	16	
MBP+eosinophils	N= 17 71.2±17.4	N= 13 110.0±32.1	NS
NE+neutrophils	N= 14 141.7±27.8	N= 12 158.1±29.9	NS
CD68+ macrophages	N= 11 47.8±24.8	N= 6 75.7±39.5	NS
CD3+ lymphocytes	N= 17 38.6±11.9	N= 11 28.3±12.8	NS
Tryptase+mast cells	N= 16 95.7±17.8	N= 8 88.24±32.2	NS
Subbasement membrane thickness (µm)	N= 17 6.8±0.3	N= 16 8.3±0.4	0.015
Smooth muscle area (%)	N= 16 9.67±1.0	N= 15 15.12±2.21	0.02
<i>Sputum (n)</i>	18	12	
Total white cell count (× 10 ⁶)	3.62±1.09	1.95±0.69	NS
Macrophages (%)	43.28±7.10	44.57±8.6	NS
Lymphocytes (%)	3.47±1.28	4.33±1.55	NS
Neutrophils (%)	49.98±6.44	40.72±7.75	NS
Eosinophils (%)	3.44±1.54	10.44±6.08	NS
<i>BAL (n)</i>	20	19	
Total cell count (× 10 ⁶)	7.08±0.96	5.76±0.68	NS
Macrophage (%)	97.09±0.89	93.24±1.11	0.007
Lymphocyte (%)	1.35±0.36	2.48±0.62	NS
Neutrophils (%)	0.97±0.31	2.61±0.5	0.018
Eosinophils (%)	0.62±0.3	1.76±0.65	NS

n, number of subjects; MBP, major basic protein; NE, neutrophil elastase; BAL, bronchoalveolar lavage. Biopsy cell counts expressed as cells/mm².

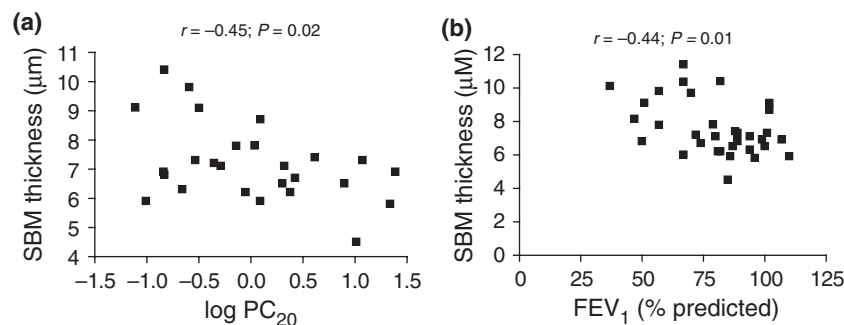


Fig. 4. Relationship of provocative concentration of methacholine causing a 20% fall in FEV₁ (PC₂₀) to subbasement membrane (SBM) thickness (a), and of post-bronchodilator forced expiratory volume in 1 s (FEV₁) to SBM thickness (b) in patients with severe and non-severe asthma.

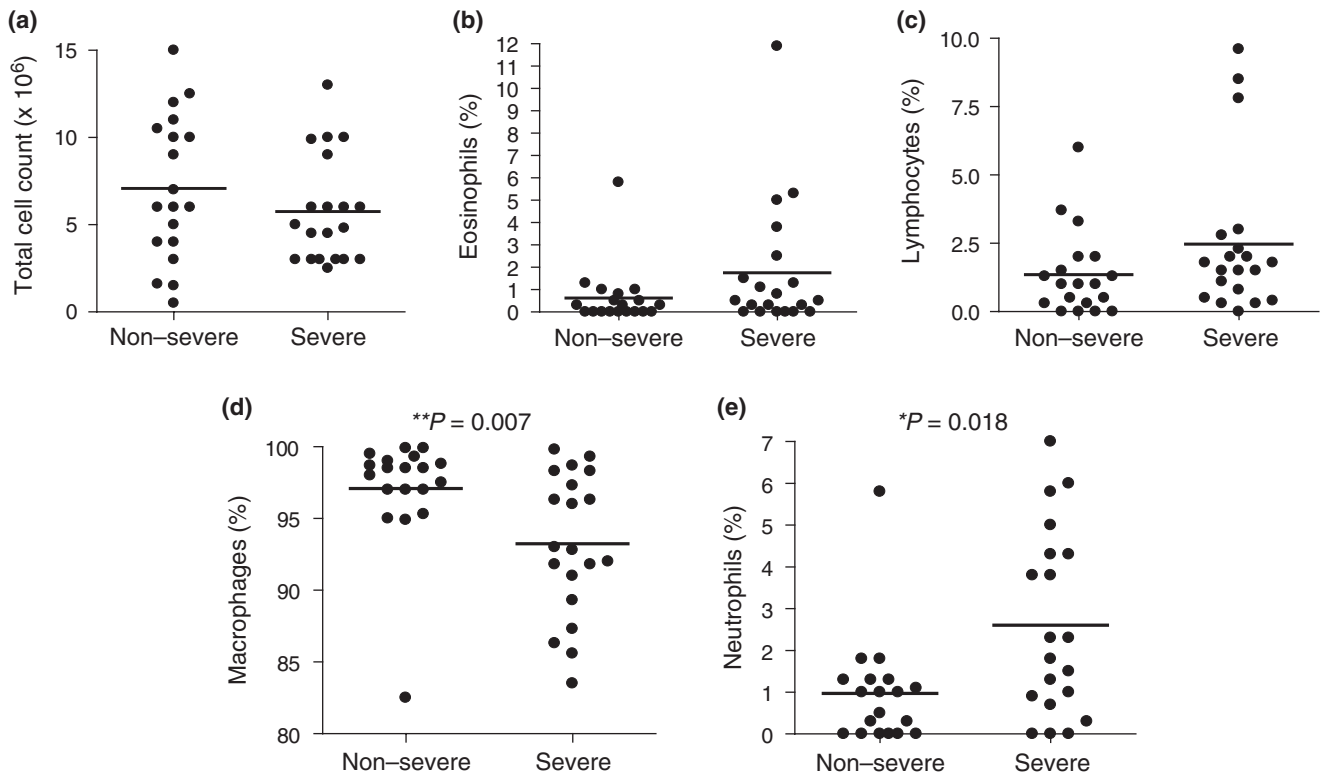


Fig. 5. Individual cell counts from bronchoalveolar lavage fluid: total cell counts (a), eosinophils (b), lymphocytes (c), macrophages (d) and neutrophils (e).

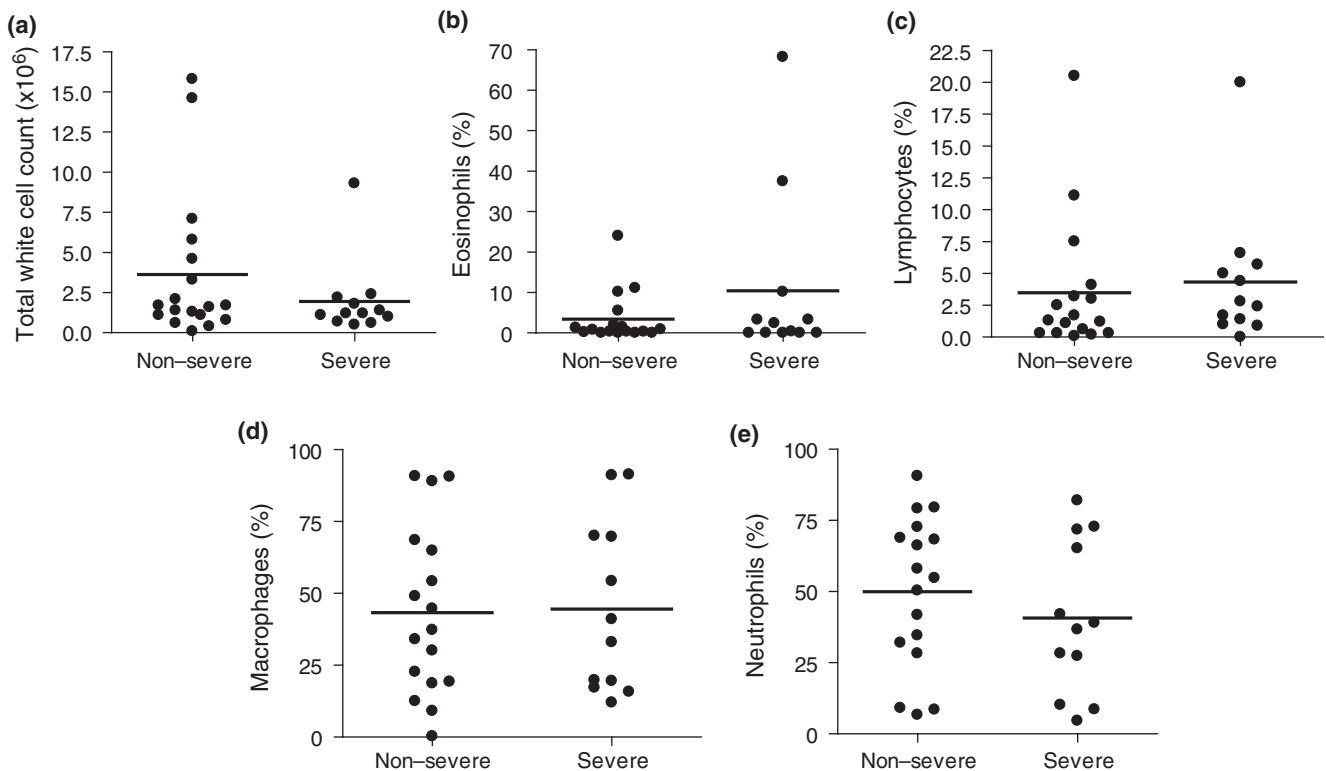


Fig. 6. Individual cell counts from induced sputum in the same order as shown for Fig. 5.

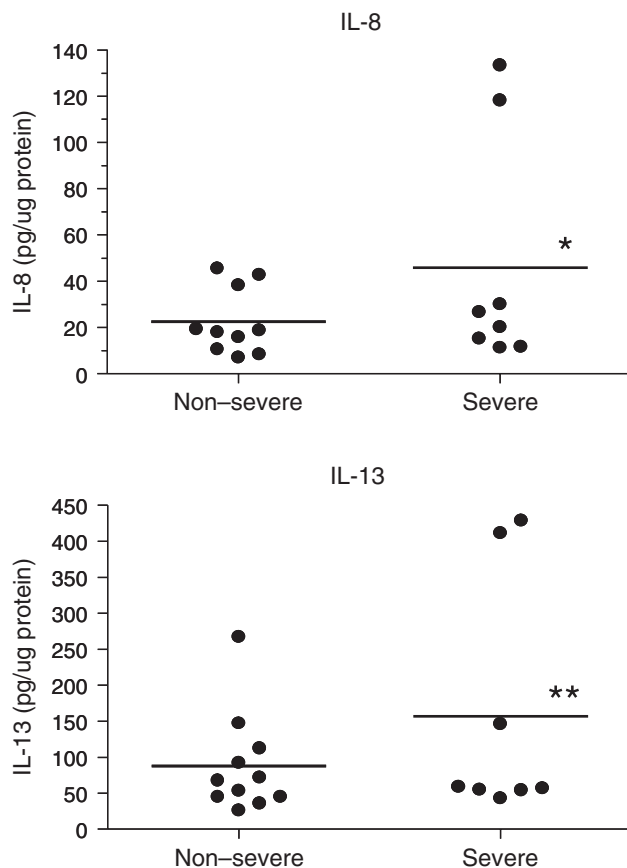


Fig. 7. Individual levels of IL-8 and IL-13 in induced sputum supernatants of patients with non-severe and severe asthma. * $P < 0.01$, ** $P < 0.02$.

any significant inverse correlation between eosinophil and neutrophil counts in our patients. Larger groups of patients with severe asthma are needed to address this issue of neutrophilic inflammation. It would be fair to conclude that despite the higher doses of CS in severe asthma, there is still persistent inflammation at similar levels as found in non-severe asthma. Of particular note, the persistence of greater ASM area and SBM thickness in severe asthma taking high-dose inhaled and oral CS would suggest that these abnormalities may not respond to CS therapy.

The other caveat in the interpretation of the inflammatory cell data is the relatively lower numbers of sections in which we could count inflammatory cell numbers. This was mainly due to technical factors of staining procedure. However, our data appear to be valid for the inflammatory cell counts, as they were in a similar range that was reported in our group in a group of patients with asthma presenting as a predominant chronic cough [15]. In the same study, we had a group of non-asthmatic normal controls, and it is clear that the two current groups of asthma patients all had significantly elevated numbers of eosinophils because, in normal non-asthmatic subjects,

the baseline level of eosinophils in biopsies is relatively low [15].

Measurement of inflammatory cells in induced sputum was also not possible in all patients, particularly in severe asthma. While we were able to obtain sputum in 18 out of 20 non-severe patients, this was only successful in 12 out of 20 severe asthma patients, mainly because no sputum was produced or the inhalation of hypertonic saline was not tolerated long enough in those with significant airflow obstruction. By contrast, we were able to obtain BAL from most asthmatics, although the procedure itself induced significant asthma worsening in two patients with severe asthma.

We found significantly higher levels of the cytokines IL-8 and IL-13 in the sputum supernatants of patients with severe asthma compared with non-severe asthma, data that are in agreement with previous studies [5, 17]. However, we were unable to measure levels of cytokines in unconcentrated BAL from the same patients, with levels at the limit or below the limit of detection of the assay used. This indicates that sputum supernatants may be a better medium than BAL to detect cytokine levels.

Taken together, these results indicate that airway wall remodelling may be an important differentiating factor between severe and non-severe asthma at the level of the airways submucosa. The role of increased subepithelial fibrosis and of airway smooth muscle mass in the pathogenesis of severe asthma is unclear. The extracellular matrix constituents of the fibrosis in severe asthma is not known, but previous studies of mild asthmatic patients indicated that there was increased deposition of Type III collagen, fibronectin and tenascin in the SBM [18, 19]. The increase in SBM thickness found in patients with severe asthma compared with non-severe asthma has been reported by previous investigators [10, 12, 20]. However, others have not reported this increase [21], and the reason behind these conflicting data is unclear, perhaps reflecting degrees of severity of asthma being studied. Similar to the report of Bourdin *et al.* [12], we also found a negative correlation between SBM thickness and post-bronchodilator FEV₁. The correlation of this thickness with airway hyperresponsiveness indicates that the extracellular matrix may influence airway contractility, and increased contractility of asthmatic airway smooth muscle cells has been recently demonstrated when these cells were embedded in a collagen matrix gel [22]. The increase in airway smooth muscle mass in asthma deaths has been attributed to both hyperplasia and hypertrophy [23], although in severe asthma, there is evidence of only hypertrophy [10]. Further studies will be needed to determine the mechanisms underlying this increase in subepithelial fibrosis and ASM mass in severe asthma.

In summary, severe asthma is different from non-severe asthma by increased airway wall remodelling rather than by differences in inflammatory cell numbers. Future

studies should confirm these findings in larger cohorts of patients with severe asthma, and well as establishing the pathophysiological abnormalities in the airways of severe asthma patients and should develop ways of detecting these changes non-invasively.

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