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The effects of steroid therapy on inflammatory cell subtypes in asthma

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Abstract

Rationale: Airway inflammation in asthma is heterogeneous, with different phenotypes. The inflammatory cell phenotype is modified by corticosteroids and smoking. Steroid therapy is beneficial in eosinophilic asthma (EA), but evidence is conflicting regarding non-eosinophilic asthma (NEA).

Objectives: To assess the inflammatory cell phenotypes in asthma after eliminating potentially confounding effects; to compare steroid response in EA versus NEA; to investigate changes in sputum cells with inhaled corticosteroid (ICS).

Methods: Subjects undertook ICS withdrawal until loss of control or 28 days. Those with airway hyper-responsiveness (AHR) took inhaled fluticasone 1000µg daily for 28+ days. Cut points were ≥/<2% for sputum eosinophils and ≥/<61% for neutrophils.

Results: After steroid withdrawal (n=94), 67% were eosinophilic, 31% paucigranulocytic, and 2% mixed; there were no neutrophilic subjects. With ICS (n=88), 39% were eosinophilic, 46% paucigranulocytic, 3% mixed and 5% neutrophilic. Sputum neutrophils increased (19.3% to 27.7%, p=0.024). Treatment response was greater in EA for symptoms (p<0.001), quality of life (p=0.012), AHR (p=0.036) and exhaled nitric oxide (F_ENO) (p=0.007). Lesser but significant changes occurred in NEA (i.e. paucigranulocytic asthma). F_ENO was the best predictor of steroid response in NEA for AHR (AUC 0.810), with an optimum cut-point of 33ppb.

Conclusions: After eliminating the effects of ICS and smoking, we were unable to identify a neutrophilic phenotype in our patients with moderate stable asthma. ICS use led to phenotype misclassification. Steroid responsiveness was greater in EA, but the absence of eosinophilia did not indicate absence of steroid response. In NEA this was best predicted by baseline F_ENO.

(*Word count 250)*

Key words: asthma, inflammatory cell, eosinophil, neutrophil, phenotype

Introduction

Asthma phenotypes may be described both clinically and pathologically, and are heterogeneous. Increasing emphasis is being given to categorising patients in relation to inflammatory phenotype using induced sputum analysis. This may provide insights into both the natural history [1] and the potential for treatment response [2]. Four subtypes have been identified: eosinophilic, neutrophilic, paucigranulocytic or mixed cellularity, depending on the presence or absence of sputum eosinophils and/or neutrophils [3]. A simpler classification provides for two categories: eosinophilic asthma (EA) and non-eosinophilic asthma (NEA).

Eosinophilic asthma is associated with atopy and IgE-mediated eosinophilic inflammation. In general, corticosteroid therapy provides for benefits in patients with EA [4,5]. It is considered to be the commonest pathological subtype, although this perception is not strictly justified. In one review, the prevalence of EA ranged from 88% to as low as 22% of cases [6].

NEA has been demonstrated in persistent asthma of all grades of severity [1,3,7-9]. In these studies neutrophil predominance has been identified. Neutrophilic inflammation has also been demonstrated in severe asthma [10], and in acute exacerbations [11]. In contrast to EA, there is conflicting evidence regarding the efficacy of inhaled corticosteroids (ICS) in NEA. Some studies have shown NEA to be relatively steroid unresponsive [5,9,12,13]. Conversely, other studies have shown similar degrees of steroid responsiveness in both EA and NEA [14,15].

Several important background issues may influence the accuracy of induced sputum cell counts and hence phenotype classification. Firstly, there is the possibility that the inflammatory cell phenotype varies longitudinally over time. We will not address this issue further in this paper. Secondly, there are the potential effects of factors such as steroid use and cigarette smoking. Concurrent use of ICS therapy is potentially critical. Ideally patients should be steroid-free, but because withdrawing ICS carries risk, it has been avoided in the majority of studies. The effects of steroid may also be relevant in neutrophilic asthma. Corticosteroid is known to prolong the survival of functional neutrophils, at least *in vitro* [16], and thus the results of studies in which neutrophil predominance is reported may have been confounded [1,3,7,8,10,11]. Smoking may also lead to increased sputum neutrophilia [17]. Other potential confounders include age [18], air pollution [19], occupation [20], high grade exercise [21], recent viral respiratory infection [22], bronchiectasis [8], and gastro-oesophageal reflux [23].

Our study aims were threefold: to assess the prevalence of eosinophilic, neutrophilic, mixed, and paucigranulocytic patients with asthma after eliminating modifying factors; to compare the therapeutic responses to ICS in EA versus NEA phenotypes; and to investigate any changes in inflammatory phenotype classification which might occur with ICS therapy. The study was carried out in patients in whom ICS treatment was withdrawn and later recommenced, and in whom cigarette smoking was excluded. It was conducted in the context of obtaining run-in data for larger ongoing clinical trials in which establishing inflammatory phenotype and steroid responsiveness were pre-requisites (Australian New Zealand Clinical Trials Registry ACTRN12606000531516, ACTRN12606000488505).

Methods

A more detailed description of the methods is found in the online supplement.

Patients

Patients aged between 18 and 75 with stable persistent asthma were enrolled. Exclusion criteria included: respiratory infection in preceding 4 weeks; >10 pack-year smoking history

or smoking in the previous 3 months; use of oral prednisolone in previous 3 months; other pulmonary disease or significant co-morbidity.

Study design
The study comprised two phases: run-in and steroid withdrawal (phase 1) and a trial of steroid (phase 2) as shown in table 1.

Baseline measurements and withdrawal of inhaled corticosteroid (Phase 1)
At the initial visit, all participants gave written informed consent. Demographic and medical data were obtained. Measurements included peak expiratory flow (PEF), fraction of exhaled nitric oxide (F_ENO), spirometry and bronchodilator response, skin prick testing and total IgE. Subjects received a symptom diary, peak flow meter, an emergency prednisolone supply, albuterol inhaler, large volume spacer and a contact details card. Diaries were completed for two weeks while subjects continued on their usual medications and they recorded morning and evening peak flow, bronchodilator use, night wakening with asthma symptoms, and asthma symptom score.

Following the run-in, individualized criteria for "loss of control" (LOC) were generated (table E1, online supplement). Subjects completed validated questionnaires to assess asthma control (Asthma Control Questionnaire (ACQ) and Asthma Control Test (ACT)) and quality of life (Asthma Quality of Life Questionnaire with standardized activities (AQLQ)). ICS and long-acting β-agonists were withdrawn, and subjects were reviewed regularly by telephone contact until either LOC or 28 days, whichever came sooner, at which time the next visit was scheduled. LOC was deemed to have occurred when one or more of the pre-set criteria were met. At LOC, testing took place over two to four consecutive days: firstly, to define the inflammatory phenotype in a population which was steroid-free; and secondly, to make baseline measurements against which the effectiveness of the trial of steroid could be measured. Where LOC was deemed to be due to respiratory infection, the patient was excluded. Testing included: hypertonic saline challenge +/- methacholine challenge +/- spirometry with bronchodilator response. Subjects proceeded to phase 2 if they had: a provocative dose of hypertonic saline causing a 15% fall in forced expiratory volume in one second (FEV_1) of less than 12 mls (PD_{15}<12mls hypertonic saline), a provocative dose of methacholine causing a 20% fall in FEV_1 of less than 8µmol (PD_{20}<8µmol methacholine), or ≥12% improvement in FEV_1 post bronchodilator.

Trial of steroid (Phase 2)
Patients were given fluticasone (Flixotide, GlaxoSmithKline, Greenford, UK) 1000µg daily via a spacer for 28+ days during which they completed the daily diary. ACQ, ACT, AQLQ, F_ENO, spirometry, sputum induction and adenosine monophosphate (AMP) challenge were carried out in sequential order before and after treatment. Steroid responsiveness was defined as one or more of the following: ≥12 increase in FEV_1; ≥0.5 point decrease in ACQ; ≥2 doubling dose increase in PC_{20}AMP; ≥40% decrease in F_ENO.

Study procedures
After airway hyper-responsiveness (AHR) to hypertonic saline was measured, induced sputum was immediately collected, the whole sample was processed and a non-squamous cell differential obtained. All cell counts were read and agreed by two trained observers. A cut-point of ≥2% eosinophils was used to define EA, and <2% to define NEA [24]. Eosinophilic, neutrophilic, mixed, and paucigranulocytic inflammation were defined using cut-points of ≥/<2% for sputum eosinophils [24] and ≥/<61% for sputum neutrophils [3]. A panel of cytokines

as well as neutrophil elastase (NE) were measured in sputum supernatant. Standardized protocols were used for methacholine and AMP challenges. Ethical approval was obtained from the Lower South Regional Ethics Committee.

Statistical analysis

Comparisons between EA and non-EA subjects were made before and after steroid withdrawal using unpaired t-tests and Mann-Whitney U tests for continuous data, and Chi-squared tests for categorical data. F_ENO, PD_{15} hypertonic saline, $PC_{20}AMP$, % eosinophils, % bronchoepithelial cells, % lymphocytes, and total cell counts, were analyzed after logarithmic transformation. A comparison of steroid responsiveness between EA and NEA patients was by mixed model analysis of continuous variables. Chi-squared tests were used to compare proportions of EA and NEA patients with clinically significant improvements in ACQ, FEV_1 and $PC_{20}AMP$ after treatment using the pre-defined cut-points. Receiver operating characteristic (ROC) curves were used to compare predictors of steroid responsiveness, and sensitivities, specificities, positive and negative predictive values and accuracy were calculated.

Results

Of a total of 165 individuals that were screened, 94 had objective evidence of airway hyper-responsiveness or reversible airflow obstruction. Eighty eight individuals completed the trial of inhaled fluticasone (figure 1). Baseline characteristics for the 94 participants are shown in table 2. Seventy-four were taking regular ICS. Of these 52 (70%) lost control after steroid withdrawal.

After steroid withdrawal, sixty five subjects (69%) were classified as eosinophilic (EA) and 29 (31%) as non-eosinophilic (NEA). Those classified as EA were more often taking ICS (EA: 86%, NEA: 62%, p=0.008) and at higher doses (EA: 856+/-620µg; NEA: 397+/-478µg, p=0.001) at baseline. Of the 56 with EA taking regular ICS, 46 (82%) experienced loss of control after steroid withdrawal compared to 6 of 18 (33%) with NEA (p<0.001) (figure E1, online supplement).

Changes in symptoms and lung function with steroid treatment

Table 3 shows results for ACQ, ACT, AQLQ, PEF, FEV_1, $PC_{20}AMP$ and F_ENO after steroid withdrawal (table E2, online supplement) and subsequently after treatment for 28+ days with inhaled fluticasone. The effect of fluticasone was significant for all measured parameters except mean morning peak flows. The response to treatment was significantly greater in EA compared to NEA for all parameters except mean morning peak flows and FEV_1.

Using predetermined cut-points for clinically significant change, steroid responsiveness was significantly more frequent in EA than in NEA for ACQ (p=0.001), FEV_1 (p<0.001), $PC_{20}AMP$ (p=0.008) and F_ENO (p<0.001) (table 4). Analyses using cut-points of 1% and 3% to define EA provided similar results although the differences between EA and NEA were of lesser magnitude (tables E8 and E9, online supplement).

Changes in inflammatory cell phenotypes with steroid treatment

After steroid withdrawal 63 (67%) subjects were eosinophilic, 29 (31%) were paucigranulocytic, and 2 (2%) were mixed. There were no neutrophilic subjects (table 5). Similar results were obtained using cut-points of ≥/<1% or ≥/<3% for sputum eosinophils. After fluticasone, 32 (51%) of the original eosinophilic subjects remained eosinophilic while 22 (35%) became paucigranulocytic, 2 (3%) became mixed and 2 (3%) became neutrophilic.

Twenty one (72%) of the original paucigranulocytic subjects remained paucigranulocytic while 4 (14%) became eosinophilic and 3 (10%) became neutrophilic. Thus a total of 5 subjects (5%) were designated neutrophilic following steroid. One of the original mixed subjects did not change while the other became eosinophilic.

After steroid withdrawal there was no difference in the neutrophil proportions in EA versus NEA (table E2, online supplement). As expected, eosinophils decreased significantly in EA with fluticasone from 17.9 (14.1-22.8) % to 3.6 (2.3-5.8) %, but did not change in NEA ($p<0.001$). However, for all subjects there was an increase in sputum neutrophils with fluticasone, from 19.3 (15.7-22.9) % to 27.7 (23.1-32.4) % ($p=0.024$), but this was not significantly different between EA and NEA (table 6). There was a significant relationship between age and % sputum neutrophils ($r=0.33$, $p<0.001$), which persisted with fluticasone ($r=0.26$, $p=0.014$). There were no significant correlations between sputum cell counts and BMI.

At loss of control or 28 days after steroid withdrawal, EA was characterised by higher levels of IL-1β ($p=0.033$), IL-5 ($p<0.001$), IL-6 ($p<0.001$), IL-8 ($p=0.014$), and IL-10 ($p=0.014$) in sputum supernatant compared to NEA (table E3, online supplement). With fluticasone, IL-8 and neutrophil elastase increased significantly (from 622.9 (352.2-698.4) pg/ml to 2207.2 (936.8-4925.9) pg/ml, median (IQR), $p<0.001$, and from 101.8 (78.2-134.0) ng/ml to 160.0 (123.7-208.5) ng/ml, median (IQR), $p<0.001$, respectively).

Predictors of steroid responsiveness in NEA

In order to explore whether objective measurements at baseline or LOC might predict steroid responsiveness in the NEA group, ROC analyses were carried out. Areas under the curve (AUCs) are shown in table 7 and relevant comparisons are shown in tables E4, E5 and E6 (online supplement). FEV_1 and PC_{20}AMP were predictors of an increase in FEV_1. F_ENO was the best predictor of steroid response as defined by an improvement in PC_{20}AMP (table E4), with an optimum cut-point of 33ppb (table E7). None of the measured parameters were able to predict an improvement in ACQ.

Discussion (see online supplement for further comments)

In this study we have clarified important issues pertaining to the classification of inflammatory phenotypes in asthma and their relationship to steroid response. Our principal findings are: firstly, after steroid withdrawal, we were unable to identify a neutrophilic phenotype in our population of patients; secondly, ICS treatment was associated with increased airway neutrophils and a switch to a neutrophilic phenotype in some patients; thirdly, although steroid responsiveness is greater in EA, it is not exclusive to this phenotype but also occurs in NEA; and finally, F_ENO may be used to predict steroid response in NEA.

Our finding of the absence of a neutrophilic phenotype after steroid withdrawal and, with steroid, of a significant increase in the proportion of sputum neutrophils (~10%) and in the number of patients with neutrophilic asthma, has significant implications. It may be that in previous studies reporting inflammatory phenotypes, the results are not strictly accurate because patients were receiving corticosteroid [1,3,7,8,10,11]. While discontinuing treatment may be impractical, the modifying effect of steroid should be taken into account. The same is true for studies including smokers [9,] in whom neutrophilia is more marked. In one study, the sputum neutrophil count was 23% in non-smokers and 47% in smokers [17]. The neutrophilic phenotype is more widely reported in severe asthma [10]. This may in part reflect the extent to which patients have greater steroid exposure. Other factors such as exercise [21] and respiratory

infection [22] may influence sputum cells; these were excluded from our study. The demonstration of neutrophilic inflammation [1,3,7-9,10] has prompted the suggestion that the neutrophil may be a key effector cell in NEA [25] and thus a target for novel therapies . Such speculation may be less appropriate.

Our findings are consistent with evidence that steroid prolongs survival of functional neutrophils [16]. In human studies, neutrophilia is greater in steroid-dependent intractable asthma (SDIA) compared to non-SDIA [26]. With steroid, neutrophils in endobronchial biopsies increased (from 43.5 to 150.8 cells/mm^2, $p<0.001$) [27], and after prednisolone (from 76 to 140 cells/mm^2, $p=0.05$) [28]. The greater increase in neutrophils seen in these studies may reflect that different tissues were sampled. It is not clear whether neutrophils associated with steroid exposure are activated or are innocent bystanders. We have shown that IL-8, a neutrophil chemo-attractant, is increased in sputum supernatant after steroid, consistent with the finding that IL-8 mRNA expression is increased after oral methylprednisolone [29]. Our finding of a significant increase in neutrophil elastase with treatment suggests that resident neutrophils retain functional capacity.

Our study demonstrated that although steroid response was significantly greater in EA, it was not unique to that phenotype. Although the presence of eosinophilia indicates greater likelihood of steroid response in airways disease [4], our results suggest that the absence of eosinophilia does not imply the absence of treatment response. A proportion of NEA subjects showed significant improvements with fluticasone: reduced symptoms, 46%; increased airway calibre, 14%; reduced AHR, 43% (table 4). This picture is consistent with data from Godon et al. [14]: 15/46 patients with sputum eosinophils <1% demonstrated improvement in AHR with fluticasone. Similarly, in another study, the benefits of systemic steroid treatment were independent of the pre-treatment sputum eosinophil count [15]. In contrast, in a randomized trial using mometasone, Berry et al. reported no significant steroid-related improvements in symptoms or AHR in non-eosinophilic subjects [13]. Overall, the inconsistencies in these data suggest that it is probably unwise to categorise NEA as a distinct steroid-unresponsive entity. Perhaps the distinction between EA and NEA based on a specific cut-point for sputum eosinophils is a false one. NEA (i.e. paucigranulocytic rather than neutrophilic) may be a milder form of the same pathology but without eosinophil trafficking into the airway lumen. Thus the presence of eosinophils is informative, but their absence is not necessarily a reliable indicator regarding steroid response.

Predicting steroid responsiveness in airways disease is important. Not surprisingly, in our study, FEV_1 was the best predictor of increased airflow with treatment in both EA and NEA (table 7). No pre-treatment test was helpful in predicting improved symptoms in NEA. However, F_ENO was the most useful predictor of steroid response in NEA as measured by a reduction in AHR, with a cut-point of 33ppb giving the best predictive accuracy. As far as we are aware this finding is novel, and surprising given that F_ENO is regarded as a surrogate marker for airway eosinophilia [30]. The high predictive value of F_ENO for improved AHR with fluticasone in NEA reinforces earlier findings regarding the predictive value of F_ENO measurements in steroid-naïve subjects [31].

A limitation of our study is that a placebo-controlled design was not used for the steroid trial. This was for ethical reasons. Seventy percent of patients taking ICS lost control after treatment withdrawal. It would have been inappropriate to treat these patients with placebo for up to 28 days beyond the point of LOC. An alternative would have been to select only patients able to tolerate ICS withdrawal. However, this would have resulted in selection bias;

only patients with mild asthma would have been eligible and the data obtained would have been less generalizable. It is possible that, because the trial was not placebo-controlled, the significance of the changes in symptoms is questionable. Similarly, although we cannot discount that individual inflammatory cell profiles might regress to the mean or change with time, in the absence of exacerbations or changes in treatment, this seems unlikely [32].

Our method for sputum analysis was to use the whole sample rather than selected sputum plugs. Although caution is required when making comparisons between this and other studies in which the alternative method is used, whole sample processing is well validated and provides comparable results [24]. Pizzichini et al. [33] confirmed that there are no significant differences in the cell proportions when comparing selected versus residual sputum. *A priori* we chose a cut point of ≥2% eosinophils to define EA [24]. This was based on the work of Belda [34] and Spanavello [35], who showed that in normal subjects the mean plus 2 standard deviations for sputum eosinophils is approximately 2%. In fact we re-analysed our data using cut-points of 1% and 3%, but this had no major effect on overall results. In fact, a cut-point of 2.3% was best for predicting changes in FEV_1 (table E10, online supplement).

In conclusion, steroid therapy contributes to increased airway neutrophilia as well as reduced eosinophilia. Thus, the inflammatory cell phenotypes reported in previous studies may be inaccurate, influenced by the effects of steroid exposure and smoking. While identifying the eosinophilic phenotype is important, it is not definitive for determining the response to steroid therapy. Modified responses to corticosteroid may still occur in patients with NEA (i.e. paucigranulocytic asthma), and can be predicted using F_ENO measurements.

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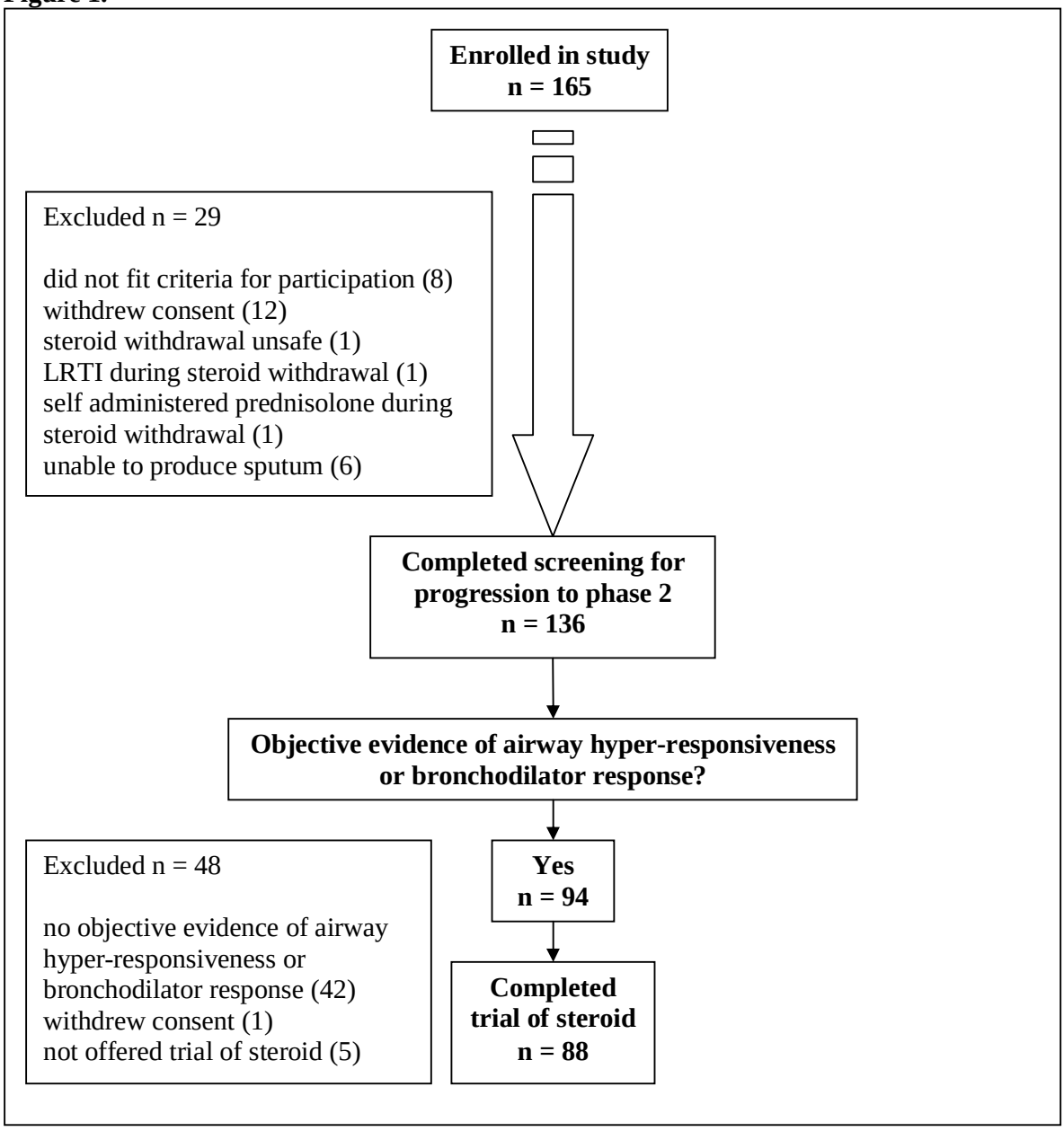
Table 1.

	Phase 1 – RUN-IN / STEROID WITHDRAWAL						Phase 2 – STEROID TRIAL			
	14 day run-in		Steroid withdrawal till LOC or 28 days	Screening for progression to Phase 2[†]			Fluticasone 500µg b.d. 28+ days			
Visit	**1**[*]	**2**		**3.1**	**(3.2)**	**(3.3)**	**3.4**		**4.1**	**4.2**
Clinical assessment	x									
Skin Prick Testing	x									
F_ENO	x						x			x
Spirometry	x			x	x	x	x		x	x
Bronchodilator response	x					x			x	
Blood tests incl. IgE	x									
ACQ, ACT, AQLQ		x		x					x	
H. S. challenge				x						
Sputum induction				x					x	
M. challenge					x					
AMP challenge							x			x
Diary		x	x					x		

Legend for Table 1: Study Plan. [*] steroid naïve subjects progressed immediately from visit 1 to visit 3.1. [†] see text for further details regarding progression through screening visits and criteria for progression to phase 2. Abbreviations: ACQ = Asthma Control Questionnaire; ACT = Asthma Control Test; AQLQ = Asthma Quality of Life with standardized activities; AMP = Adenosine monophosphate; F_ENO = fraction of exhaled nitric oxide; H. S. = Hypertonic saline; IgE = immunoglobulin E; LOC = loss of control; M. = Methacholine.

Figure 1.



Legend for Figure 1: Consort diagram outlining the selection and treatment allocation of patients. Abbreviations: LRTI = lower respiratory tract infection.

Table 2.

	All (n=94)	*EA (n=65)*	*NEA (n=29)*	**p (EA v NEA)**
Age (years)	43 (13)	43 (12)	43 (16)	0.980
Male[*]	34 (36%)	24 (37%)	10 (34%)	0.820
Age at onset (years)	17 (18)	19 (18)	13 (17)	0.134
Ex-smokers[*]	25 (27%)	19 (29%)	6 (21%)	0.387
Atopic[*]	74 (80%)	50 (77%)	24 (86%)	0.335
On ICS[*]	74 (79%)	56 (86%)	18 (62%)	**0.008**
ICS dose (µg daily) [†]	714 (616)	856 (620)	397 (478)	**0.001**
ICS dose (µg daily) [†‡]	700 (200-1000)	1000 (400-1000)	200 (0-600)	**<0.001**
On LABA/ combined ICS+LABA[*]	30 (32%)	24 (37%)	6 (21%)	0.119
ACQ	0.9 (0.6)	0.9 (0.7)	0.9 (0.6)	0.714
FEV_1 % predicted	88 (16)	88 (16)	88 (16)	0.902
FEV_1 % change post-bronchodilator	10 (9)	10 (10)	9 (7)	0.501
F_ENO (ppb) [**]	27.8 (24.0-32.2)	29.6 (24.7-35.6)	24.1 (18.9-30.7)	0.204

Legend for Table 2: Baseline characteristics of all subjects, subsequently categorized as eosinophilic asthma (EA) and non-eosinophilic asthma (NEA). Results presented as mean (SD) or n (%) unless otherwise stated. Unpaired t-tests were used for comparisons unless otherwise stated. [*]Analyzed by Chi-squared tests. [†]Beclomethasone equivalents: 1µg beclomethasone = 1µg budesonide = 0.5µg fluticasone. [‡]Analyzed by Mann-Whitney U test and results presented as median (IQR). [**]Analyzed after logarithmic transformation and results presented as geometric mean (95% confidence intervals). Significant p-values in bold. Abbreviations: ICS = inhaled corticosteroid; LABA = long-acting beta-agonist; ACQ = asthma control questionnaire; FEV_1 = forced expiratory volume in 1 second; F_ENO = fraction of exhaled nitric oxide.

Table 3.

	All (n=88)				*EA (n=60)*			*NEA (n=28)*			
	before	**after**	**Δ**	**p**	**before**	**after**	**Δ**	**before**	**after**	**Δ**	**p (Rx effect in EA v NEA)**
ACQ	1.6 (0.9)	0.7 (0.6)	-0.9 (0.9)	**<0.001**	1.9 (0.9)	0.7 (0.6)	-1.1 (0.9)	1.0 (0.6)	0.7 (0.5)	-0.3 (0.5)	**<0.001**
ACT	18 (4)	21 (3)	3 (4)	**<0.001**	17 (4)	21 (3)	4 (4)	20 (4)	21 (3)	1 (3)	**0.011**
AQLQ	5.7 (0.9)	6.4 (0.6)	0.7 (0.8)	**<0.001**	5.5 (1.0)	6.4 (0.6)	0.9 (0.9)	6.2 (0.6)	6.4 (0.6)	0.2 (0.4)	**0.012**
am PEF (L/min)	376 (98)	415 (117)	10 (10) *	0.132	372 (95)	416 (116)	12 (10) *	390 (108)	410 (125)	5 (8) *	0.583
FEV_1 (L)	2.36 (0.83)	2.86 (0.83)	26 (25) *	**0.002**	2.22 (0.78)	2.85 (0.80)	34 (26) *	2.68 (0.86)	2.87 (0.90)	7 (7)*	0.094
$PC_{20}AMP$ (mg/ml)†	18.7 (11.8-29.6)	157.4 (100.9-245.5)	3.1 (2.9) ‡	**<0.001**	10.2 (6.0-17.4)	141.9 (80.9-248.9)	3.8 (3.0) ‡	53.9 (26.9-108.0)	188.5 (90.3-393.7)	1.8 (2.2) ‡	**0.036**
F_ENO (ppb)†	44.2 (37.8-51.5)	20.6 (18.3-23.1)	-25.1 (-57.9.-6.0) **	**<0.001**	55.2 (46.6-65.4)	21.6 (18.8-24.9)	-35.9 (-62.5,-15.4) **	27.7 (21.7-35.2)	18.5 (15.2-22.6)	-7.3 (-19.0.-0.7) **	**0.007**

Legend for Table 3: Changes in symptoms (ACQ, ACT, AQLQ), lung function (morning PEF, FEV_1), airway hyper-responsiveness ($PC_{20}AMP$) and airway inflammation (F_ENO) in eosinophilic asthma (EA) and non-eosinophilic asthma (NEA) after inhaled fluticasone (1000µg daily) given for 28+ days. Presented as mean (SD) unless otherwise stated. Changes (Δ) expressed as: absolute changes for ACQ, ACT, AQLQ and F_ENO; * percentage changes for PEF and FEV_1; and ‡ doubling dose changes for $PC_{20}AMP$. Analyzed using mixed model analysis. †Analyzed after logarithmic transformation and results presented as geometric mean (95% C.I.). ** Change (Δ) in F_ENO presented as median (IQR). Abbreviations: ACQ = asthma control questionnaire; ACT = Asthma Control Test; AQLQ = Asthma Quality of Life with standardized activities; am PEF = morning peak flow rate; FEV_1 = forced expiratory volume in 1 second; $PC_{20}AMP$ = provocation concentration of adenosine monophosphate causing a 20% fall in FEV_1; F_ENO = fraction of exhaled nitric oxide.

Table 4.

	All		*EA*		*NEA*		*p*
	+ve	***-ve***	***+ve***	***-ve***	***+ve***	***-ve***	***EAvNEA***
ACQ	61/88 (69%)	27/88 (31%)	48/60 (80%)	12/60 (20)	13/28 (46%)	15/28 (54%)	**0.001**
FEV_1	52/88 (59%)	36/88 (41%)	48/60 (80%)	12/60 (20%)	4/28 (14%)	24/28 (86%)	**<0.001**
$PC_{20}AMP$	48/77 (62%)	29/77 (38%)	36/49 (73%)	13/49 (27%)	12/28 (43%)	16/28 (57%)	**0.008**
F_ENO	53/87 (61%)	34/87 (39%)	44/59 (75%)	15/59 (25%)	9/28 (32%)	19/28 (68%)	**<0.001**

Legend for Table 4: Categorical analysis of steroid response in eosinophilic asthma (EA) and non-eosinophilic asthma (NEA) using predetermined cut-points for improvements in symptoms, lung function, airway hyper-responsiveness and airway inflammation. A positive response occurred in those subjects whose change for a given endpoint with treatment exceeded the following cut-points: ACQ ≥0.5 point decrease; FEV_1 ≥12% increase; $PC_{20}AMP$ ≥2 doubling dose increase; and F_ENO ≥40% decrease. Results presented as proportions (%). Comparisons between EA and NEA analyzed using Chi-squared tests. Not all subjects were able to undergo AMP challenge for safety reasons. Abbreviations: ACQ = asthma control questionnaire; FEV_1 = forced expiratory volume in 1 second; $PC_{20}AMP$ = provocation concentration of adenosine monophosphate causing a 20% fall in FEV_1; F_ENO = fraction of exhaled nitric oxide.

Table 5.

Phenotype at LOC or 28 days after steroid withdrawal		*Phenotype after fluticasone 1000µg daily for 28+ days*				
		Eosinophilic	***Paucigranulocytic***	***Mixed***	***Neutrophilic***	***NA***
Eosinophilic	63 (67%)	32 (51%)	22 (35%)	2 (3%)	2 (3%)	5 (8%)
Paucigranulocytic	29 (31%)	4 (14%)	21 (72%)	0 (0%)	3 (10%)	1 (3%)
Mixed	2 (2%)	1 (50%)	0 (0%)	1 (50%)	0 (0%)	0 (0%)
Neutrophilic	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
All	94 (100%)	37 (39%)	43 (46%)	3 (3%)	5 (5%)	6 (6%)

Legend for Table 5: Sputum phenotype at loss of control (LOC) or 28 days after steroid withdrawal and after fluticasone 1000µg daily for 28+ days. Results expressed as n (%). Eosinophilic = eosinophils ≥2%, neutrophils <61%; paucigranulocytic = eosinophils <2%, neutrophils <61%; mixed = eosinophils ≥2%, neutrophils ≥61%; neutrophilic = eosinophils <2%, neutrophils ≥61%. NA = sputum sample not available after trial of steroid.

Table 6.

	All (n=88)			*EA (n=60)*		*NEA (n=28)*		
	before	**after**	**p**	**before**	**after**	**before**	**after**	**p (Rx effect by phenotype)**
*Total cell count ($x10^6$/ml) **	1.2 (0.9-1.5)	0.9 (0.8-1.1)	0.439	1.4 (1.1-1.9)	1.0 (0.7-1.2)	0.7 (0.5-1.0)	0.8 (0.6-1.2)	0.117
Viability (%)	88.8 (86.8-90.8)	91.1 (89.5-92.8)	**0.047**	89.2 (87.0-91.4)	90.7 (88.7-92.8)	87.9 (83.7-92.0)	92.0 (89.2-94.8)	0.357
Squamous cells (%)	18.1 (15.2-21.0)	21.3 (17.8-24.8)	0.552	15.7 (12.3-19.0)	21.9 (17.6-26.2)	23.3 (18.0-28.6)	20.0 (14.0-26.0)	0.056
*Eosinophils (%)**	5.8 (3.9-8.7)	2.0 (1.4-3.0)	**0.001**	17.9 (14.1-22.8)	3.6 (2.3-5.8)	0.5 (0.4-0.8)	0.6 (0.3-1.0)	**<0.001**
Neutrophils (%)	19.3 (15.7-22.9)	27.7 (23.1-32.4)	**0.024**	20.2 (15.8-24.5)	30.6 (25.3-35.9)	17.5 (11.1-23.9)	21.6 (12.8-30.4)	0.323
Macrophages (%)	41.0 (36.4-45.6)	47.3 (42.3-52.3)	0.052	37.8 (32.3-43.2)	42.6 (37.0-48.2)	47.9 (39.6-56.2)	57.3 (48.1-66.5)	0.528
*Bronchoepithelial cells (%)**	11.5 (9.0-14.7)	9.6 (7.7-12.0)	**0.033**	8.3 (6.2-11.1)	9.8 (7.7-12.4)	23.1 (16.5-32.3)	9.2 (5.7-15.0)	**0.002**
*Lymphocytes (%)**	1.1 (0.8-1.4)	0.6 (0.4-0.7)	**0.004**	1.3 (1.0-1.7)	0.5 (0.4-0.7)	0.7 (0.4-1.1)	0.6 (0.4-0.9)	**0.023**

Legend for Table 6: Changes in sputum cells in eosinophilic asthma (EA) and non-eosinophilic asthma (NEA) after inhaled fluticasone (1000µg daily) for 28+ days. Results presented as mean (95% CI). Analyzed using mixed model analysis. * Analyzed after logarithmic transformation and results presented as geometric mean (95% CI). Significant p values are in bold.

Table 7.

AUC

Outcomes / Predictors	EA			NEA		
	$\Delta PC_{20}AMP$	**ΔFEV_1**	**ΔACQ**	**$\Delta PC_{20}AMP$**	**ΔFEV_1**	**ΔACQ**
FEV_1 (baseline)	0.308	0.613	0.254	0.305	**0.849**	0.387
FEV_1 (LOC)	0.426	**0.818**	0.411	0.315	**0.865**	0.379
BD response	0.435	0.609	0.549	0.429	0.616	0.470
$PD_{15}HS$	0.538	0.597	0.558	0.500	0.401	0.641
$PC_{20}AMP$	0.686	0.691	**0.710**	0.656	**0.708**	0.646
F_ENO	**0.778**	0.699	**0.727**	**0.810**	0.354	0.631

Legend for Table 7: Areas under the curve for receiver operator characteristic analyses in which measurements of FEV_1 at baseline, FEV_1 at loss of control or 28 days after steroid withdrawal (LOC), change in FEV_1 with bronchodilator at baseline, airway hyper-responsiveness as measured by PD_{15} hypertonic saline and by PC_{20} adenosine monophosphate, and F_ENO, were used as predictors. The outcomes were: change in airway hyper-responsiveness as measured by $PC_{20}AMP$ ($\Delta PC_{20}AMP$), change in FEV_1 (ΔFEV_1), and change in ACQ (ΔACQ) following 28+ days of inhaled fluticasone treatment in 60 patients with eosinophilic asthma (EA) (49 for $PC_{20}AMP$) and 28 patients with non-eosinophilic asthma (NEA). An AUC of >0.7 is considered significant. Abbreviations: AUC = area under the curve; FEV_1 = forced expiratory volume in 1 second; BD response = bronchodilator response; $PD_{15}HS$ = provocation dose of hypertonic saline causing a 15% fall in FEV_1; $PC_{20}AMP$ = provocation concentration of adenosine monophosphate causing a 20% fall in FEV_1; F_ENO = fraction of exhaled nitric oxide; ACQ = asthma control questionnaire.

The effects of steroid therapy on inflammatory cell subtypes in asthma

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Online Supplement

Methods (online supplement)

Patients

Patients were aged between 18 and 75 years with a history of stable persistent asthma were enrolled. The primary source for patients was the Otago Respiratory Research Unit Database which holds details of over 1000 individuals who have participated in one or more previous studies within the Unit. Other sources included outpatient chest clinic, respiratory ward, pulmonary function laboratory, and primary care. In addition, posters displayed around the hospital inviting interested individuals to contact the Unit at which time their suitability could be assessed. Exclusion criteria were: respiratory tract infection in preceding 4 weeks; >10 pack year smoking history or smoking in the previous 3 months; use of oral prednisolone in previous 3 months; history of life threatening asthma; forced expiratory volume in one second (FEV_1) <50% predicted; other pulmonary disease; significant co-morbidity likely to influence the conduct of the study; pregnancy and breast feeding.

Study design

This study comprised two phases; run-in and steroid withdrawal (phase 1) and a trial of steroid (phase 2) as shown in table 1.

Baseline measurements and withdrawal of inhaled corticosteroid (Phase 1)

At the initial visit, information regarding the study was provided and all participants gave written informed consent. Demographic and medical data were obtained, a brief examination performed (including height, weight, pulse, BP and chest exam) and suitability for the study confirmed. Subjects performed three peak flow manoeuvres to ensure correct technique. Fraction of exhaled nitric oxide (F_ENO) measurements were made in accordance with ERS/ATS guidelines [1] using a chemiluminescence analyser (NiOX, Aerocrine, Solna,

Sweden) and a flow rate of 50mL/s. Spirometry was performed using a rolling seal spirometer (SensorMedics Corporation, Yorba Linda, CA) in accordance with ATS/ERS guidelines and bronchodilator response was assessed 15 minutes after inhalation of 400µg of albuterol via a large volume spacer (Volumatic, GlaxoSmithKline, Greenford, UK) [2]. Skin prick testing was performed to positive and negative controls, cat pelt, grass mix, and house dust mite (Hollister-Stier Laboratories LLC Spokane, WA). Atopy was defined as a wheal of >2mm greater than the negative control, to one or more allergens. Blood samples were taken for routine haematology, biochemistry and total IgE.

Subjects were provided with a symptom diary, peak flow meter (Breath-Alert, Medical Developments International, Springvale, Victoria, Australia), an emergency prednisolone supply, albuterol inhaler (100µg, Ventolin, GlaxoSmithKline, Greenford, UK), large volume spacer and an emergency contact details card. Diaries were completed for a period of two weeks while subjects continued on their usual asthma medications. Recordings were: morning and evening peak flow (best of three), bronchodilator use, night wakening with asthma symptoms and asthma symptom score. The score described overall respiratory symptoms (shortness of breath, cough, wheeze etc.) over the previous 24 hours on a scale from 0 to 5: 0 = no symptoms at all, 1 = symptoms for one short period during the day, 2 = symptoms for two or more short periods of the day, 3 = symptoms for most of the day which did not affect normal daily activities, 4 = symptoms for most of the day which affected normal daily activities and 5 = symptoms so severe that patient could not go to work or perform daily activities [3].

Following the two week run-in, diaries were reviewed and the following calculated: mean morning and evening peak flows, mean number of puffs of bronchodilator taken per 24 hours,

and mean number of nights woken with asthma symptoms per week. Individualized criteria for "loss of control" (LOC) were generated using a modification of criteria developed by Jones et al. [4] (see table E1, online supplement). Subjects completed validated questionnaires to assess asthma control (Asthma Control Questionnaire (ACQ) [5] and Asthma Control Test (ACT) [6,7]), and quality of life (Asthma Quality of Life Questionnaire with standardized activities (AQLQ) [8]). Inhaled corticosteroids (ICS) and long-acting β-agonists were withdrawn, and subjects were reviewed regularly by telephone contact until either LOC or 28 days, whichever came sooner, at which time the next visit was scheduled. LOC was deemed to have occurred when one or more of the pre-set criteria were met.

At LOC, testing took place over two to four consecutive days: firstly, to define the inflammatory phenotype in a steroid-free population; and secondly, to make baseline measurements against which the effectiveness of the trial of steroid could be measured. Where LOC was deemed to be due to respiratory infection, the patient was excluded. Testing included hypertonic saline challenge +/- methacholine challenge +/- spirometry with bronchodilator response, and subjects proceeded to phase 2 if they had one of the following: a provocative dose of hypertonic saline causing a 15% fall in forced expiratory volume in one second (FEV_1) of less than 12 mls (PD_{15}<12mls hypertonic saline) [9], a provocative dose of methacholine causing a 20% fall in FEV_1 of less than 8μmol (PD_{20}<8μmol methacholine) [10], or ≥12% improvement in FEV_1 post bronchodilator [11].

Trial of steroid (Phase 2)

Patients were given fluticasone (Flixotide, GlaxoSmithKline, Greenford, UK) 1000μg daily by inhalation via a spacer for 28+ days during which they completed the daily diary. ACQ, ACT, AQLQ, F_ENO, spirometry, sputum induction and adenosine monophosphate (AMP)

challenge were carried out in sequential order before and after treatment. Steroid responsiveness was defined as one or more of the following: ≥12 increase in FEV_1 [11]; ≥0.5 point decrease in ACQ [12]; ≥2 doubling dose increase in PC_{20}AMP [10,13]; and ≥40% decrease in F_ENO [14]. Other end-points were mean morning peak flow over the last 7 days of the trial, ACT and AQLQ scores.

Study procedures

Airway hyper-responsiveness (AHR) to hypertonic saline was assessed using a standardized protocol [9]. An ultrasonic nebuliser (ultra-neb 2000, DeVilbiss, Somerset, PA) and a Hans-Rudolph two-way non-rebreathing valve mouthpiece (No.2700 Hans Rudolph Inc., Shawnee, KA) were used to deliver 4.5% saline. Doses were given for 30 seconds, 1, 2, 4 and 8 minutes, with spirometry performed at baseline and one minute after each dose. The challenge was discontinued when a 15% fall in FEV_1 occurred or after a cumulative inhalation time of 15.5 minutes. AHR was defined as a 15% fall in FEV_1 from baseline after a cumulative provocation dose of 4.5% hypertonic saline (PD_{15}) <12mls [9].

Immediately thereafter, induced sputum was collected, and the whole sample processed and a cell differential obtained from 400 non-squamous cells using a standardized method [15]. All cell counts were read and agreed by two trained observers. A cut-point of ≥2% was used to define EA, <2% to define NEA [16]. Eosinophilic, mixed, neutrophilic and paucigranulocytic inflammation were defined using cut-points of ≥/<2% for sputum eosinophils [16] and ≥/<61% for sputum neutrophils [17].

Sputum supernatant was stored at -80°C for later cytokine analysis. A panel of cytokines (interleukins 1β, 4, 5, 6, 8, 10, 12; interferon-γ, and tumour necrosis factor-α) were measured

in sputum supernatant using Bio-Plex cytokine multiplex bead-based assays kits (Bio-Rad Laboratories, Hercules, CA) [18]. Neutrophil elastase (NE) was measured in sputum supernatant using the InnoZyme Human Neutrophil Elastase Immunocapture Activity Assay Kit (Merck Ltd., Auckland, New Zealand) [19].

Methacholine challenge was performed using a modification of the protocol described by Yan et al [20]. Spirometry was performed at baseline and one minute after delivery of 0.9% saline and doubling doses of methacholine administered via a nebuliser controlled by a calibrated dosimeter (Morgan, Kent, UK) which administered doses of methacholine ranging between 0.069µmol and 17.626µmol. The challenge was discontinued when a 20% fall from post-saline FEV_1 occurred or after the maximum dose of methacholine had been given. The provocation dose causing a 20% fall in FEV_1 from post-saline baseline (PD_{20}) was calculated by interpolation of the dose response curve. Significant AHR was defined as a PD_{20} of <8 µmol [10].

AMP challenge was performed using a standardized protocol [21]. Spirometry was performed at baseline, 1 minute after 0.9% saline and 1 and 3 minutes after doubling doses of AMP ranging from 0.59mg/ml to 300mg/ml delivered by a nebuliser connected to a breath-activated dosimeter (Morgan, Kent, UK). The lower of the two FEV_1 measurements was recorded for each dose. The test was terminated on reaching a 20% fall in FEV_1 or after the maximum dose of AMP had been administered. The provocative concentration causing a 20% fall in FEV_1 (PC_{20}AMP) was derived by linear interpolation of the dose-response curve. AMP challenges in which a 20% fall in FEV_1 was not achieved were assigned a PC_{20}AMP of 1200mg/ml.

Ethical considerations and patient safety

Ethical approval was obtained from the Lower South Regional Ethics Committee. To ensure safety during phase 1, subjects were provided with individualized action plans, prednisolone tablets for emergency use, and an emergency contact details card. Subjects were contacted at regular intervals during the period off treatment and in addition subjects had 24-hour access to a study investigator via cell-phone and the hospital paging system. LOC criteria included "presence of distressing or intolerable asthma symptoms" so that phase 1 could be terminated at the patient's request regardless of peak flow measurements if necessary.

Statistical analysis

Statistical analysis was carried out using SPSS version 16.0. Comparisons between eosinophilic and non-eosinophilic subjects were made before and after steroid withdrawal using unpaired t-tests and Mann-Whitney U tests for continuous data, and Chi-squared tests for categorical data. F_ENO, PD_{15} hypertonic saline, $PC_{20}AMP$, % eosinophils, % bronchoepithelial cells, % lymphocytes, and total cell counts were analyzed after logarithmic transformation. Change in $PC_{20}AMP$ with treatment was expressed as doubling dose shift using the formula: $[\log_{10}(\text{pre-treatment } PC_{20}) - \log_{10}(\text{post-treatment } PC_{20})] / \log_{10}2$ [22]. A comparison of steroid responsiveness between EA and NEA patients was by mixed model analysis of continuous variables. Chi-squared tests were used to compare proportions of EA and NEA patients with clinically significant improvements in ACQ, FEV_1 and $PC_{20}AMP$ after treatment using the pre-defined cut-points. Receiver operating characteristic (ROC) curves were used to compare different predictors of steroid responsiveness [23] and sensitivities, specificities, positive and negative predictive values and accuracies were calculated. P values of <0.05 were accepted as statistically significant. Results are expressed as mean (standard deviation) unless otherwise stated.

Discussion

The reduction in airway responsiveness to AMP with steroid, notably in the NEA group, may reflect an effect of steroid on mast cell mediated inflammation, a feature which was not assessed in our study. Berry et al. have shown that mast cell numbers in airway smooth muscle are increased in both EA and NEA compared to controls [24], and this is associated with AHR [25]. AMP challenge induces release of mast-cell derived mediators [26] and hence may be a measure of mast-cell related hyper-responsiveness. Both inhaled [27] and oral [28] steroid treatment result in a reduction in mucosal mast cells numbers, and may also act by inhibiting mast cell degranulation [29]. Thus, it may be that the improvement in PC_{20}AMP seen in NEA as well as EA is related to changes in mast cell numbers and function.

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Tables (online supplement)

Legend for Table E1: Criteria for loss of control (LOC) defined as the presence of *one or more* of the listed clinical features.

Legend for Figure E1: Steroid withdrawal and subsequent loss of control (LOC) in eosinophilic asthma (EA) and non-eosinophilic asthma (NEA). Results presented as n (%). Analyzed by Chi-squared tests. Significant p values are in bold.

Legend for Table E2: Symptoms (ACQ, ACT, AQLQ), lung function (PEF, FEV_1), airway hyper-responsiveness ($PD_{15}HS$, $PC_{20}AMP$), and airway inflammation (F_ENO and sputum cells), in eosinophilic asthma (EA) and non-eosinophilic asthma (NEA) at loss of control or 28 days after steroid withdrawal. Results presented as mean (SD) unless otherwise stated. Comparisons between EA and NEA at loss of control are by unpaired t tests unless otherwise stated. * Proportion of subjects with uncontrolled asthma using definitions of ACQ $\geq$1.5 [30] and ACT $\leq$19 [7]; presented as n (%) and analyzed using Chi-squared tests. † Analyzed after logarithmic transformation and results presented as geometric mean (95% confidence intervals). Significant p values are in bold. Abbreviations: ACQ = asthma control questionnaire; ACT = asthma control test; AQLQ = asthma quality of life questionnaire with standardized activities; PEF = morning peak expiratory flow; FEV_1 = forced expiratory volume in 1 second; $PD_{15}HS$ = provocation dose of hypertonic saline causing a 15% fall in FEV_1; $PC_{20}AMP$ = provocation concentration of adenosine monophosphate causing a 20% fall in FEV_1; F_ENO = fraction of exhaled nitric oxide.

Legend for Table E3: Sputum supernatant mediators in eosinophilic asthma (EA) and non-eosinophilic asthma (NEA) at loss of control or 28 days after steroid withdrawal. All

mediators measured in pg/ml unless otherwise stated. Analyzed using Mann-Whitney U test. Data expressed as median (IQR). Significant p values are in bold.

Legend for Table E4: Area under the curve (AUC) comparisons (z scores, p values) for FEV_1 at baseline, FEV_1 at loss of control or 28 days after steroid withdrawal (LOC), change in FEV_1 with bronchodilator at baseline, airway hyper-responsiveness as measured by PD_{15} hypertonic saline and by PC_{20} adenosine monophosphate, and F_ENO, as predictors of steroid response as defined by increase in $PC_{20}AMP$ ≥2 doubling doses following 28+ days of inhaled fluticasone (1000µg daily) in 28 subjects with non-eosinophilic asthma. Significant p values are in bold. Abbreviations: AUC = area under the curve; FEV_1 = forced expiratory volume in 1 second; BD response = bronchodilator response; $PD_{15}HS$ = provocation dose of hypertonic saline causing a 15% fall in FEV_1; $PC_{20}AMP$ = provocation concentration of adenosine monophosphate causing a 20% fall in FEV_1; F_ENO = fraction of exhaled nitric oxide; ACQ = asthma control questionnaire.

Legend for Table E5: Area under the curve (AUC) comparisons (z scores, p values) for FEV_1 at baseline, FEV_1 at loss of control or 28 days after steroid withdrawal (LOC), change in FEV_1 with bronchodilator at baseline, airway hyper-responsiveness as measured by PD_{15} hypertonic saline and by PC_{20} adenosine monophosphate, and F_ENO, as predictors of steroid response as defined by increase in FEV_1≥12% doses following 28+ days of inhaled fluticasone (1000µg daily) in 28 subjects with non-eosinophilic asthma. Significant p values are in bold. Abbreviations: AUC = area under the curve; FEV_1 = forced expiratory volume in 1 second; BD response = bronchodilator response; $PD_{15}HS$ = provocation dose of hypertonic saline causing a 15% fall in FEV_1; $PC_{20}AMP$ = provocation concentration of adenosine

monophosphate causing a 20% fall in FEV_1; F_ENO = fraction of exhaled nitric oxide; ACQ = asthma control questionnaire.

Legend for Table E6: Area under the curve (AUC) comparisons (z scores, p values) for FEV_1 at baseline, FEV_1 at loss of control or 28 days after steroid withdrawal (LOC), change in FEV_1 with bronchodilator at baseline, airway hyper-responsiveness as measured by PD_{15} hypertonic saline and by PC_{20} adenosine monophosphate, and F_ENO, as predictors of steroid response as defined by decrease in ACQ ≥0.5 following 28+ days of inhaled fluticasone (1000µg daily) in 28 subjects with non-eosinophilic asthma. Significant p values are in bold. Abbreviations: AUC = area under the curve; FEV_1 = forced expiratory volume in 1 second; BD response = bronchodilator response; $PD_{15}HS$ = provocation dose of hypertonic saline causing a 15% fall in FEV_1; $PC_{20}AMP$ = provocation concentration of adenosine monophosphate causing a 20% fall in FEV_1; F_ENO = fraction of exhaled nitric oxide; ACQ = asthma control questionnaire.

Legend for Table E7: Sensitivities, specificities, positive predictive values, negative predictive values and accuracy of different F_ENO cut-points for predicting increase in $PC_{20}AMP$≥2 doubling doses with inhaled fluticasone 1000µg daily for 28+ days in 28 subjects with non-eosinophilic asthma. Optimum cut-points for F_ENO in bold. Abbreviations: F_ENO = fraction of exhaled nitric oxide; $PC_{20}AMP$ = provocation concentration of adenosine monophosphate causing a 20% fall in FEV_1.

Legend for Table E8: Changes in symptoms (ACQ, ACT, AQLQ), lung function (mean PEF, FEV_1), airway hyper-responsiveness ($PC_{20}AMP$) and airway inflammation (F_ENO) in eosinophilic asthma (EA) and non-eosinophilic asthma (NEA) after inhaled fluticasone (1000µg daily) given for 28+ days. A cut-point of ≥/<3% for sputum eosinophils was used to

define EA and NEA. Presented as mean (SD) unless otherwise stated. Changes (Δ) expressed as: absolute changes for ACQ, ACT, AQLQ and F_ENO; * percentage changes for PEF and FEV_1; and ‡ doubling dose changes for $PC_{20}AMP$. Analyzed using mixed model analysis. †Analyzed after logarithmic transformation and results presented as geometric mean (95% C.I.). ** Change (Δ) in F_ENO presented as median (IQR). Abbreviations: ACQ = asthma control questionnaire; ACT = Asthma Control Test; AQLQ = Asthma Quality of Life with standardized activities; am PEF = morning peak flow rate; FEV_1 = forced expiratory volume in 1 second; $PC_{20}AMP$ = provocation concentration of adenosine monophosphate causing a 20% fall in FEV_1; F_ENO = fraction of exhaled nitric oxide.

Legend for Table E9: Categorical analysis of steroid response in eosinophilic asthma (EA) and non-eosinophilic asthma (NEA) using predetermined cut-points for improvements in symptoms, lung function, airway hyper-responsiveness and airway inflammation. A cut-point of ≥/<3% for sputum eosinophils was used to define EA and NEA. A positive response occurred in those subjects whose change for a given endpoint with treatment exceeded the following cut-points: ACQ ≥0.5 point decrease; FEV_1 ≥12% increase; $PC_{20}AMP$ ≥2 doubling dose increase; and F_ENO ≥40% decrease. Results presented as proportions (%). Comparisons between EA and NEA analyzed using Chi-squared tests. Not all subjects were able to undergo AMP challenge for safety reasons. Abbreviations: ACQ = asthma control questionnaire; FEV_1 = forced expiratory volume in 1 second; $PC_{20}AMP$ = provocation concentration of adenosine monophosphate causing a 20% fall in FEV_1; F_ENO = fraction of exhaled nitric oxide.

Legend for Table E10. Sensitivity, specificity, positive and negative predictive values, and overall accuracy of different sputum eosinophil cut-points for predicting increase in FEV_1

≥12% following 28+ days of inhaled fluticasone (1000µg daily) in 88 subjects. (Area under the curve = 0.880). Optimum cut-point for sputum eosinophils in bold.

Table E1.

Criteria for loss of control
Average morning peak flow < 90% of baseline over the last week
2 consecutive morning or evening peak flows <80% of baseline in last week
Mean daily bronchodilator use of 4 puffs more than during run-in over the last week
Night wakening with asthma symptoms on 2 nights per week more than during run-in
Presence of distressing or intolerable asthma symptoms

Figure E1.

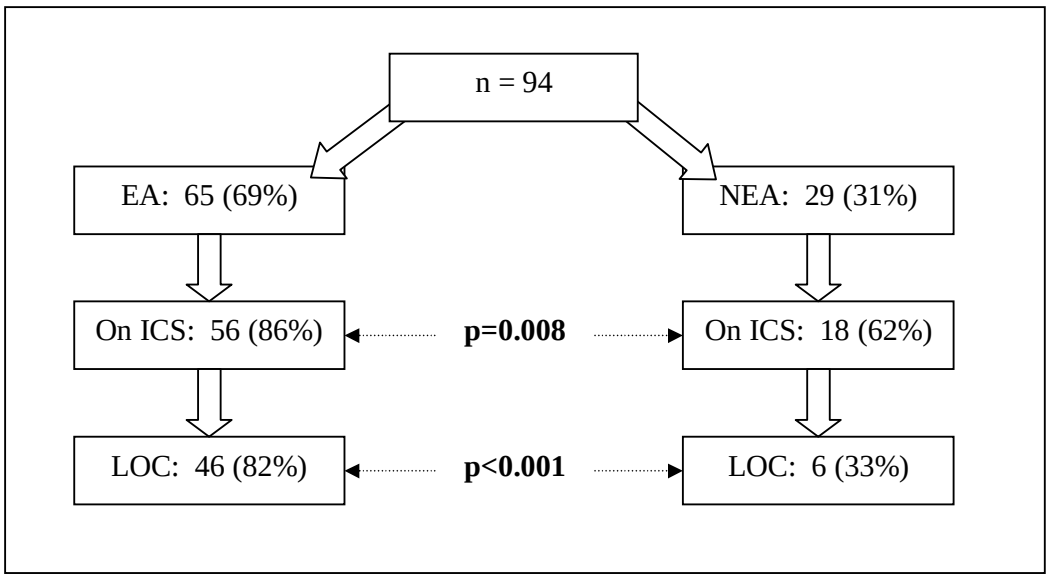

Table E2.

	All (n=94)	EA (n=65)	NEA (n=29)	p EA v NEA
ACQ	1.6 (0.9)	1.8 (0.9)	1.0 (0.6)	**<0.001**
*ACQ ≥1.5**	50 (53%)	44 (68%)	6 (21%)	**<0.001**
ACT	18 (4)	17 (4)	19 (3)	**0.016**
*ACT ≤19**	57 (61%)	44 (68%)	13 (45%)	**0.036**
AQLQ	5.7 (0.9)	5.5 (1.0)	6.1 (0.6)	**0.004**
PEF (L/min)	374 (95)	369 (92)	390 (104)	0.420
FEV_1 (L)	2.37 (0.82)	2.22 (0.76)	2.70 (0.85)	**0.007**
$PD_{15}HS$ (ml) †	6.6 (5.1-8.7)	5.4 (3.9-7.4)	10.1 (6.5-15.7)	**0.027**
$PC_{20}AMP$ (mg/ml) †	20.3 (12.9-31.8)	11.6 (6.8-19.8)	58.3 (29.3-116.0)	**0.001**
F_ENO (ppb) †	42.0 (36.0-48.9)	50.4 (42.1-60.2)	28.1 (22.2-35.5)	**<0.001**
Eosinophils (%) †	6.0 (4.1-8.8)	17.6 (14.1-22.1)	0.5 (0.4-0.8)	**<0.001**
Neutrophils (%)	19.1 (16.8)	20.1 (16.8)	17.0 (17.0)	0.420
Lymphocytes (%) †	1.0 (0.8-1.3)	1.3 (1.0-1.7)	0.6 (0.4-1.0)	**0.005**

Table E3.

Mediator	ALL (n=91)	EA (n=62)	NEA (n=29)	p
IL-1β	37.7 (19.4-74.0)	48.7 (21.0-78.7)	26.3 (12.1-40.2)	**0.033**
IL-4	0.5 (0.3-1.0)	0.5 (0.3-1.0)	0.5 (0.3-0.9)	0.997
IL-5	2.9 (1.2-6.0)	4.1 (2.0-8.8)	1.9 (0.9-2.7)	**<0.001**
IL-6	10.7 (4.8-24.7)	15.9 (7.6-51.8)	4.4 (1.2-12.3)	**<0.001**
IL-8	622.9 (352.2-698.4)	641.9 (381.4-717.6)	579.5 (171.0-647.1)	**0.014**
IL-10	0.8 (0.5-1.2)	0.9 (0.6-1.4)	0.6 (0.4-0.9)	**0.014**
IL-12	1.7 (0.9-3.0)	1.8 (0.9-3.1)	1.5 (0.9-2.7)	0.506
IFN-γ	21.8 (11.5-35.5)	24.5 (12.3-39.1)	20.1 (10.0-34.2)	0.226
TNF-α	7.0 (4.0-18.4)	8.6 (4.3-23.9)	6.4 (3.4-12.0)	0.114
NE (ng/ml)	101.8 (78.2-134.0) (n=87)	103.9 (84.0-134.0) (n=59)	90.6 (73.5-134.6) (n=28)	0.393

Table E4.

	AUC	*FEV_1 (baseline)*	*FEV_1 (LOC)*	*BD response*	*$PD_{15}HS$*	*$PC_{20}AMP$*
FEV_1 (baseline)	0.305					
FEV_1 (LOC)	0.315	0.182				
		p=0.428				
BD response	0.429	0.773	0.717			
		p=0.220	p=0.237			
$PD_{15}HS$	0.500	1.320	1.216	0.447		
		p=0.093	p=0.112	p=0.328		
$PC_{20}AMP$	0.656	**2.910**	**2.784**	1.441	1.432	
		p=0.002	**p=0.003**	p=0.075	p=0.076	
F_ENO	**0.810**	**3.994**	**4.038**	**2.621**	**2.191**	1.100
		p<0.001	**p<0.001**	**p=0.004**	**p=0.014**	p=0.136

Table E5.

	AUC	*F_ENO*	*$PD_{15}HS$*	*BD response*	*$PC_{20}AMP$*	*FEV_1 (baseline)*
F_ENO	0.354					
$PD_{15}HS$	0.401	0.229				
		p=0.409				
BD response	0.616	1.323	0.831			
		p=0.093	p=0.203			
$PC_{20}AMP$	**0.708**	**1.888**	**2.330**	0.376		
		p=0.030	**p=0.010**	p=0.353		
FEV_1 (baseline)	**0.849**	**2.794**	**2.684**	0.982	0.953	
		p=0.003	**p=0.004**	p=0.163	p=0.170	
FEV_1 (LOC)	**0.865**	**3.217**	**2.890**	1.112	1.142	0.247
		p<0.001	**p=0.002**	p=0.133	p=0.127	p=0.402

Table E6.

	AUC	*FEV_1 (LOC)*	*FEV_1 (baseline)*	*BD response*	*F_ENO*	*$PD_{15}HS$*
FEV_1 (LOC)	0.379					
FEV_1 (baseline)	0.387	0.148				
		p=0.441				
BD response	0.470	0.565	0.515			
		p=0.286	p=0.303			
F_ENO	0.631	**1.940**	**1.852**	1.007		
		p=0.026	**p=0.032**	p=0.157		
$PD_{15}HS$	0.641	**1.917**	**1.933**	1.079	0.067	
		p=0.028	**p=0.027**	p=0.140	p=0.473	
$PC_{20}AMP$	0.646	**1.857**	**1.812**	1.101	0.099	0.039
		p=0.032	**p=0.035**	p=0.135	p=0.461	p=0.484

Table E7.

F_ENO (ppb)	*Sensitivity (%)*	*Specificity (%)*	*Positive predictive value (%)*	*Negative predictive value (%)*	*Accuracy (%)*
11.3	100.0	25.0	50.0	100.0	57.1
21.6	91.7	43.8	55.0	87.5	64.3
25.6	91.7	62.5	64.7	90.9	75.0
30.3	83.3	68.8	66.7	84.6	75.0
31.8	**83.3**	**81.2**	**76.9**	**86.7**	**82.1**
33.2	**75.0**	**87.5**	**81.8**	**82.4**	**82.1**
36.8	66.7	87.5	80.0	77.8	78.6
40.3	58.3	87.5	77.8	73.7	75.0
44.0	50.0	87.5	75.0	70.0	71.4
46.8	41.7	87.5	71.4	66.7	67.9
48.3	33.3	87.5	66.7	63.6	64.3

Table E8.

	All (n=88)				EA (n=58)			NEA (n=30)			
	before	**after**	**Δ**	**p**	**before**	**after**	**Δ**	**before**	**after**	**Δ**	**p (Rx effect in EA v NEA)**
ACQ	1.6 (0.9)	0.7 (0.6)	-0.9 (0.9)	**<0.001**	1.9 (0.9)	0.8 (0.6)	-1.2 (0.9)	0.9 (0.6)	0.7 (0.5)	-0.3 (0.5)	**<0.001**
ACT	18 (4)	21 (3)	3 (4)	**<0.001**	17 (4)	21 (3)	4 (5)	20 (3)	21 (3)	1 (3)	**0.011**
AQLQ	5.7 (0.9)	6.4 (0.6)	0.7 (0.8)	**<0.001**	5.5 (1.0)	6.4 (0.6)	0.9 (0.9)	6.1 (0.6)	6.4 (0.6)	0.3 (0.4)	**0.014**
am PEF (L/min)	376 (98)	415 (117)	10 (10) *	0.122	370 (95)	415 (117)	12 (10) *	392 (105)	413 (122)	5 (8) *	0.570
FEV_1 (L)	2.36 (0.83)	2.86 (0.83)	26 (25) *	**0.001**	2.19 (0.78)	2.84 (0.81)	35 (27) *	2.69 (0.84)	2.89 (0.89)	8 (6) *	0.087
$PC_{20}AMP$ (mg/ml)†	18.7 (11.8,29.6)	157.4 (100.9,245.5)	3.1 (2.9) ‡	**<0.001**	9.8 (5.7,17.0)	130.7 (73.6,232.1)	3.7 (3.0) ‡	51.0 (26.2,99.5)	210.4 (104.2,424.8)	2.0 (2.4) ‡	0.071
F_ENO (ppb)†	44.2 (37.8,51.5)	20.6 (18.4,23.1)	-25.1 (-57.9,-6.0) **	**<0.001**	55.3 (46.4,66.0)	21.7 (18.8,25.1)	-37.0 (-63.5,-14.9) **	28.8 (22.8,36.3)	18.6 (15.5,22.4)	-8.4 (-24.1,-1.4) **	**0.011**

Table E9.

	All		EA		NEA		p
	+ve	-ve	+ve	-ve	+ve	-ve	EAvNEA
ACQ	61/88 (69%)	27/88 (31%)	47/58 (81%)	11/58 (19%)	14/30 (47%)	16/30 (53%)	**<0.001**
FEV_1	52/88 (59%)	36/88 (41%)	46/58 (79%)	12/58 (21%)	6/30 (20%)	24/30 (80%)	**<0.001**
$PC_{20}AMP$	48/77 (62%)	29/77 (38%)	34/47 (72%)	13/47 (28%)	14/30 (47%)	16/30 (53%)	**0.023**
F_ENO	53/87 (61%)	34/87 (39%)	42/57 (74%)	15/57 (26%)	11/30 (37%)	19/30 (63%)	**<0.001**

Table E10.

Sputum eosinophils (%)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)
0.1	98	19	64	88	66
0.25	96	28	66	83	68
0.4	96	31	67	85	69
0.6	96	33	68	86	70
0.75	96	36	68	87	72
0.85	96	39	69	88	73
0.95	96	42	70	88	74
1.1	96	47	72	89	76
1.35	94	53	74	86	77
1.6	94	61	78	88	81
1.8	92	64	79	85	81
2.3	**92**	**67**	**80**	**86**	**82**
2.85	90	67	80	83	81
3.1	88	67	79	80	80
3.45	87	67	79	77	78
3.8	85	67	79	75	77
4.0	83	67	78	73	76
4.2	83	69	80	74	77
4.35	83	72	81	74	78