Responsiveness to oral prednisolone in severe asthma is related to the degree of eosinophilic airway inflammation.

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Abbreviations

ASM. Airway smooth muscle

AHR. Airway hyperresponsiveness.

BAL. Bronchoalveolar lavage.

eNO. Exhaled nitric oxide.

FEV_1. Forced expiratory volume in one second.

FVC. Forced vital capacity.

ICS. Inhaled corticosteroids.

PEFR. Peak expiratory flow rate.

Pc20. Provocation concentration that causes a 20% fall in FEV_1.

Abstract

Background

Patients with severe asthma appear relatively corticosteroid resistant. Corticosteroid responsiveness is closely related to the degree of eosinophilic airway inflammation. The extent to which eosinophilic airway inflammation in severe asthma responds to treatment with systemic corticosteroids is not clear.

Objective

To relate the physiological and inflammatory response to systemic corticosteroids in asthma to disease severity and the baseline extent of eosinophilic inflammation.

Methods

Patients with mild/moderate and severe asthma were investigated before and after two-weeks of oral prednisolone (Clintrials.gov NCT00331058 and NCT00327197). We pooled the results from 2 studies with common protocols. The US study contained 2 independent centres and the UK 1 independent centre. The effect of oral corticosteroids on FEV_1, Pc20, airway inflammation and serum cytokines were investigated. Baseline measurements were compared with healthy subjects.

Results

32 mild/moderate asthmatics, 50 severe asthmatics and 35 healthy subjects took part. At baseline both groups of asthmatics had a lower FEV_1 and Pc20 and increased eosinophilic inflammation compared to healthy subjects. The severe group had a lower FEV_1 and more eosinophilic inflammation compared to mild/moderate asthmatics. Oral prednisolone caused a similar degree of suppression of eosinophilic inflammation in all compartments in both groups of asthmatics. There were small improvements in FEV_1 and Pc20 for both mild/ moderate and severe asthmatics with a correlation between the baseline eosinophilic inflammation and the change in FEV_1. There was a

~50% reduction in the serum concentration of CXCL10 (IP-10), CCL22 (MDC), CCL17 (TARC), CCL-2 (MCP-1) and CCL-13 (MCP-4) in both asthma groups after oral corticosteroids.

Conclusions and Clinical Relevance.

Disease severity does not influence the response to systemic corticosteroids. The study does not therefore support the concept that severe asthma is associated with corticosteroid resistance. Only baseline eosinophilic inflammation was associated with the physiological response to corticosteroids, confirming the importance of measuring eosinophilic inflammation to guide corticosteroid use.

Introduction

Asthma is a common condition characterized by variable airflow obstruction in association with airway inflammation which is commonly, though not invariably eosinophilic [1]. For the majority of people with asthma their disease can be controlled with modest doses of inhaled corticosteroids (ICS) and bronchodilators. However in about 5% of people disease control is more difficult to achieve. The most common cause of poor control of asthma is sub-optimal adherence to treatment, especially with ICS [2, 3]. Other reasons for symptoms despite large amounts of treatment include treatment unresponsive lung damage (usually fixed airflow obstruction or bronchiectasis), symptoms caused by alternative diagnoses, in particular various patterns of dysfunctional breathing, and comorbidities such as obesity, obstructive sleep apnoea and psychological morbidity [4]. The complexity of difficult to control asthma means that precise definitions of what constitutes severe asthma are elusive [5]. Most definitions, however, are based on the pragmatic approach of patients who are still symptomatic despite high dose ICS and bronchodilators [6]. A treatment-based definition has its drawbacks as it is influenced by the health care system, access to treatment, and the prejudices and expertise of the responsible physician. It is also influenced by symptoms to a greater extent than other components of the disease such as the degree of lung damage, the severity of airway inflammation or the risk of severe exacerbations. The term refractory asthma has been used to encompass some of these other parameters and to exclude patients with difficult to control asthma for reasons other than physiologically severe disease [6, 7]. Because, by definition, patients with refractory (severe), asthma are not controlled on high dose steroids these patients are considered relatively corticosteroid resistant.

The concept of corticosteroid resistance in asthma has a long pedigree having been first described in the context of patients who continued to have variable airflow obstruction despite treatment with two weeks of oral corticosteroids [8, 9]. This was associated with evidence of *in vitro* corticosteroid resistance in monocytes [9]. Following this initial report there have been a large number of studies suggesting various mechanisms for corticosteroid resistance in asthma. These include defects in T

cell responses, abnormal functioning of the glucocorticoid receptor and abnormalities in chromatin remodeling [10-12]. However the concept of a corticosteroid resistant phenotype remains controversial, in part because most studies have not linked corticosteroid responsiveness to eosinophilic inflammation which, measured either directly by sputum or blood eosinophils or indirectly by exhaled nitric oxide or nitrosylated bromide, is currently the best biomarker of corticosteroid responsiveness. [13-15]. Airway hyperresponsiveness (AHR) associated with mast cell infiltration of the airway smooth muscle (ASM), which underlies variable airflow obstruction is not a corticosteroid responsive process in the absence of active eosinophilic inflammation [15-17]. Thus the defining clinical hallmark of 'corticosteroid resistance' (variable airflow obstruction which doesn't improve with systemic corticosteroids), may be due to an intrinsic abnormality in ASM physiology rather than a defect in the anti-inflammatory corticosteroid signalling pathway.

Our hypothesis is that the majority of patients with severe asthma who appear corticosteroid resistant do not respond (assuming they are taking them as prescribed), because they do not have a steroid responsive disease process, rather than because there is an inherent molecular or cellular defect in the corticosteroid signalling pathway. In order to test this hypothesis we undertook 2 studies, which had followed common protocols, to determine the extent to which the immunopathology of severe asthma in comparison to mild/moderate asthma changed in response to two weeks of high dose oral prednisolone steroids and how such changes related to lung function.

Methods

Subjects

The study population comprised male and female subjects aged 18–65 years. Subjects in the healthy group were non-smokers, in good health and did not have asthma. Subjects in the asthma groups had a physician's diagnosis of asthma and had demonstrated >12% reversibility in FEV_1 or a methacholine Pc20 of <8mg/ml, with exclusion of other significant pulmonary disease. Exclusion criteria included evidence of recent infection that could preclude participation in the corticosteroid trial; history of abnormal bruising or bleeding; concomitant medications such as aspirin, atenolol or metoprolol; and subjects who had changed asthma medication or had an asthma exacerbation within the previous month. All subjects gave written informed consent. This study was approved by local ethics review committees and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. In the UK the ethics committee was NRES Committee East Midlands – Northampton, number 05/Q2502/8. In Wake Forest the study was approved by the Wake Forest University internal review board number 4 IRB00000611. In Penn State the study was approved by the the University of Pennsylvania Institutional Review Board. Subjects were allocated to four groups: one healthy group and three groups consisting of subjects with asthma of increasing severity: intermittent, mild/moderate and severe. The GINA framework and corticosteroid use were used to help define the different groups [18]. See Supplementary Table 1.

Subjects were recruited from three sites: Wake Forest (WF) School of Medicine in North Carolina, University of Pennsylvania and Glenfield Hospital in Leicester. In total 130 subjects (55 from Leicester, 58 from Wake Forest and 17 from Pennsylvania) took part in the study. 27 subjects withdrew prematurely, 16 (21%) from the US and 11(20%) from Leicester. Of the 95 asthmatics, 13 were in the intermittent asthmatic group, 32 in the mild to moderate group and 50 in the severe group. This paper describes data from the mild to moderate and severe groups. The control group

consisted of 35 healthy subjects, 13 from Leicester, 12 from Wake Forest and 10 from Pennsylvania. A summary of the study population is presented in Table 1.

Study Design

This was an open-label, parallel-group study in healthy subjects and subjects with asthma (Clintrials.gov NCT00331058 and NCT00327197). The analysis population was drawn from two studies which with respect to the data presented here had essentially identical protocols. One was conducted in the US from 18 February 2006–7 July 2011 (GSK protocol: RES100767) and one in the UK from 2 August 2005–6 June 2011 (GSK protocol: RES100769).

Endpoints were analysis of inflammatory marker expression in the bronchial mucosa, blood, sputum and bronchoalveolar lavage (BAL) and changes in serum cytokines. Other measurements reported here were forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), daily peak expiratory flow rate (PEFR), exhaled nitric oxide (NO) and airway hyper-responsiveness (AHR) measured by inhaled methacholine challenge – (provocative concentration resulting in 20% reduction of FEV_1 (PC20)).

Subjects were screened within 28 days of study start and those with asthma entered a 2-week run-in period before undergoing treatment with prednisolone. They maintained their current asthma therapy throughout the study. Subjects were provided with diary cards to record their current asthma and concomitant medication, PEFR. Adherent subjects on perceived optimal therapy proceeded to prednisolone treatment and continued to record daily PEFR.

Baseline assessments were undertaken within 14 days prior to starting prednisolone. On Day 1, subjects with asthma underwent fibre-optic bronchoscopy and then received 0.5 mg/kg/day of oral prednisolone for 14 days (daily dose did not exceed a maximum of 40 mg). A second bronchoscopy was conducted between Day 14–16 and the last dose of prednisolone was administered on the morning of the bronchoscopy.

Healthy subjects attended a screening visit and underwent baseline assessments within 14 days of undergoing a bronchoscopy. They returned for follow-up 7–14 days following bronchoscopy and did not receive prednisolone.

Sample Collection and Analysis

Bronchial biopsies, BAL fluid and sputum were collected before and after prednisolone treatment and subjected to histopathology on bronchial biopsies and cytospins on sputum and BAL cells as previously described [15]. Twenty-nine biomarkers were analysed in the serum using a variety of assays as described (Supplementary Table 3). Limits of quantification were determined for each analyte/ assay to ensure reliable data was being obtained. There were low numbers of subjects in groups for sputum cell counts due to lost samples resulting from application of quality control parameters. The sputum sample was considered good if there were at least 200 non-squamous cells and there were no more than 40% of squamous cells and at least 50% of the sample was viable. Some of the baseline immunohistochemistry data from the Leicester cohort has been previously reported [19].

Blood samples for biomarkers were collected at screening, Day -1 and Day 14 from subjects with asthma and at screening and baseline for the healthy group. Pulmonary function assessments were conducted at the follow-up visit for asthma groups and at baseline for the healthy group. For subjects with asthma, PEFR was conducted during run-in period, day -1, day 1, day 13, day 14 and

at follow-up and screening for the healthy subjects. Exhaled NO assessments were performed at screening, Day -1, and Day 13 (exhaled NO assessments were performed at screening for healthy subjects).

Statistical Analysis

Analyses were performed testing differences prior to treatment between the asthma severity groups and change in response post-treatment across the asthma groups for all the key variables. If the endpoint of interest had a screening and a baseline record, the average was taken as the pre-treatment measure.

The baseline analysis used analysis of variance with the pre-treatment value as the response and study ID and cohort as explanatory factors. The results determined significant differences between the asthma severity groups for each endpoint. A second series of analyses was performed on each endpoint to investigate differences post treatment for each cohort. This analysis used a mixed model analysis of variance which included study identifier, asthma severity and time (pre or post) as explanatory factors and interaction between group and time. Subject identifier was included as a random effect within the model. This analysis only included subjects with both pre and post-treatment measures. The analyses were performed using SAS 9.2.

Many of the endpoints were logged to meet the normality assumption for the distribution of the residuals. In the cases where the endpoint data was logged the difference between cohorts or the difference post treatment are calculated on the means of the logged data. These results are then back transformed to make interpretation easier. The result of back transforming a difference of logs is to convert the result to a ratio. With regards to the results presented in the tables all data has been logged prior to analysis except for the pulmonary function data. Values presented in Tables 2-5 are derived from the analysis of variance. Means presented are the least squares means which are adjusted appropriately for the factors in the model.

Due to the exploratory nature of the studies many endpoints have been gathered and analysed. With so many responses we do expect to see false positives and false negatives. The results of this paper should be weighed up alongside other findings and put into context. Given the exploratory nature of the study it seems unnecessary to adjust the level of significance for all findings.

Results

Baseline findings

Both the mild/moderate and severe asthma groups had a significantly lower post-bronchodilator FEV_1 than healthy subjects and the severe group had a significantly lower FEV_1 than the mild/moderate group. Subjects in both asthma groups had a significantly lower methacholine Pc20 than healthy controls with no difference between the asthma groups (Table 1).

There was a significant increase in the basement membrane thickness in the severe group (15.1 μm) compared to healthy controls (11.8 μm). This represents a mean increase of 3.3 μm, with a 95% confidence interval of (1.06, 5.50) and p=0.0045. There was no difference between the mild/moderate group and the healthy controls with a mean value of 10.9 μm in the mild/moderate group.

There was a consistent pattern of increased eosinophilic inflammation in the severe group compared to the healthy subjects. Eosinophil counts/mm^2 were almost 3 times greater in the bronchial submucosa for severe asthmatics compared to healthy subjects (10.78, n=38 for severe asthma; vs 3.68, n=15 for healthy subjects). Blood eosinophil counts were also more than double and sputum counts were over 4 times higher in the severe asthmatic group compared to healthy subjects. Even though cell counts were low within the BAL there was still an elevated response in the severe group with percentage counts being over 5 times higher. This strong effect was also evident in the mild/moderate group with subjects showing a consistently higher level of eosinophils with

significance in all compartments except for the submucosa (Table 2). Although the eosinophilic inflammation was generally more marked in the severe group compared to the mild/moderate group these differences were not significant.

Using all subjects there was a significant correlation between the numbers of blood eosinophils and the sputum and BAL eosinophil percentages with Pearson correlation coefficients of 0.65 and 0.52 respectively giving p-values < 0.0001. Correlations with the submucosal eosinophil count were weaker. The correlation between eosinophils within the submucosa and sputum gave a correlation coefficient of 0.211(p=0.0122) and with blood a coefficient of 0.281 (p=0.0023). There was no correlation of eosinophils in the submucosa with eosinophils in BAL.

There were also significant correlations between the exhaled nitric oxide concentration and the level of eosinophils in blood, sputum and BAL with correlation coefficients of 0.574, 0.598 and 0.409 respectively and all with p-value< 0.0001. There was no correlation of eosinophils in the submucosa with exhaled nitric oxide. There were differences between the US and UK cohorts for exhaled nitric oxide particularly evident in the severe group, with a mean of 19.3ppb in the US and 32.7 in the UK.

Effect of oral corticosteroids

As shown in Table 3 there were small increases in post-bronchodilator FEV_1, % predicted FEV_1, FVC and PEFR for the mild/moderate and severe groups which showed a consistently significant improvement for the severe group. The severe group (n=46) showed an increase in % predicted FEV_1 from 73.9 pre-treatment to 77.6 post treatment (increase of 3.7) and the mild/moderate (n=23) group from 87.8 to 89.9 (increase of 2.1). There were minor non-significant improvements in Pc20 in both asthma groups with the severe group increasing from a baseline value of 0.95mg/ml to

1.41mg/ml (increase of 48.3%) and the mild/moderate group showing a smaller increase from a baseline of 1.45mg/ml to 1.99mg/ml (increase of 37%)

As shown in Table 4 there was a significant fall in the concentration of exhaled nitric oxide for both asthma groups with an average decrease of 34%. There was a highly significant reduction post corticosteroid in the number of eosinophils in the blood, and the percentage in the sputum and BAL with reductions in the severe group of 75%, 78% and 72% respectively and in the mild/ moderate group of 75%, 65% and 64% respectively. Out of the 68 subjects with severe or mild/ moderate asthma 60 (88%) of them showed a decrease in the number of eosinophils post treatment. There was also a statistically significant reduction in the number of eosinophils in the bronchial sub-mucosa with a 71% reduction in the mild/ moderate group and a 50% reduction in the severe group. There was a statistically significant increase in neutrophils for the severe group in the peripheral blood, sputum and BAL fluid. The mild/ moderate group only showed a significant increase in peripheral blood neutrophils.

The relationship between the change in FEV_1 in response to corticosteroid and baseline measurements from sputum, blood, BAL and submucosa were assessed. Correlation plots of pulmonary function responses with eosinophil responses in blood, BAL and sputum are shown in Supplementary figure 1 with the correlation coefficients presented in supplementary table 2. The severe subjects show a much stronger correlation of baseline eosinophils with change in FEV_1 than the mild/ moderate and severe subjects combined. When assessing the severe subjects alone the correlation coefficients for blood, sputum and BAL were 0.458 (n=46, p=0.0014), 0.480 (n=27, p=0.0113), 0.405 (n=41, p=0.0087) respectively, showing evidence of the relationship between high levels of baseline eosinophils and improvement in FEV_1. No correlations were found between any of the baseline eosinophil counts and change in FEV_1 in the mild/moderate group. Exhaled nitric oxide did not correlate with change in FEV_1 in either group. There were no significant correlations

between the change in any of the eosinophil counts and the change in FEV_1 in either group. There were no correlations between the baseline neutrophil count and the change in FEV_1 post corticosteroids or between the change in neutrophil counts and the change in FEV_1 in any compartment.

Other submucosal cellular and structural biomarkers

There were no consistent significant changes in the numbers of mast cells, lymphocytes or monocytes in any of the submucosal compartments including the airway smooth muscle in either the severe or mild/moderate groups. There was no change in basement membrane thickness in either asthmatic group in response to oral corticosteroids

Serum mediators

No differences in baseline concentrations of any of the cytokines tested were seen between the groups. Concentrations of the serum protein biomarkers, CXCL10 (interferon-inducible protein-10 (IP-10)), and CCL-22 (macrophage-derived chemokine (MDC)), showed very significant decreases in both asthma groups after prednisolone compared with baseline (decreases of 42% and 55%, respectively). CCL-17 (Thymus and activation-regulated chemokine (TARC)), and CCL-2 (monocyte chemotactic protein-1 (MCP-1)), also showed significant decreases of 31% and 30%, respectively in both asthma groups. There was a smaller but significant decrease of 17% in CCL-13 (MCP-4) in both asthma groups after prednisolone treatment, compared with baseline (Table 5). The observed decreases in serum protein markers were similar between the mild/moderate and severe groups. There were no notable changes in concentrations of the other protein biomarkers (data not shown). Due to the number of biomarkers investigated a Bonferroni adjustment would suggest that a more stringent p-value of $p<0.002$ should be used as a guide for assessing significance.

Safety

During the treatment period, 24 (21%) subjects reported AEs (Supplemental Table 3).

Discussion.

The two main aims of this study were firstly to determine if there were inflammatory features in the asthmatic airway which would predict the physiological and immunological response to oral corticosteroids and secondly whether we could document lack of corticosteroid responsiveness in severe asthma compared to mild to moderate asthma. We found that the only predictor of an improvement in FEV_1 in response to corticosteroids was the baseline eosinophil count. We also found that there were very similar responses to systemic corticosteroids between the mild/moderate and severe groups across a range of measures providing no evidence to support the concept of corticosteroid resistance as a general feature of severe asthma.

We identified a number of significant differences at baseline between the severe asthmatics and healthy controls including a reduced FEV_1 and methacholine Pc20 as well as increased epithelial basement membrane thickening and the presence of eosinophilic inflammation. These differences were also seen between mild/moderate asthma and healthy controls, but the eosinophilic inflammation was less marked. There were relatively few differences between the severe and mild/moderate groups in terms of baseline physiology, inflammatory markers, structural and cellular changes in the bronchial mucosa and serum mediators with only a lower FEV_1 and an increased basement thickness (consistent with a greater degree of eosinophilic inflammation), reaching significance.

We demonstrated in this study that essentially all the subjects with asthma responded to high dose oral corticosteroids as evidenced by a reduction in the peripheral blood and airway eosinophil count and a reduced concentration of several chemokines in peripheral blood, with no difference in response between severe and mild to moderate subjects. High dose oral corticosteroids also resulted in a reduction in sputum, BAL and to a lesser extent submucosal eosinophils, although there was no relationship between the change in these counts and the change in FEV_1 after corticosteroids. However the degree of baseline eosinophilia in sputum, blood and BAL did correlate with improvement in FEV_1. Together this demonstrates that the response to corticosteroids in terms of lung function in severe asthma is closely linked to the presence of active eosinophilic airway inflammation and suggests that corticosteroids are working in large part through ameliorating eosinophilic inflammation. Our data contradicts the idea that there is significant corticosteroid resistance in severe asthma suggesting the relative failure to respond to treatment with high dose ICS is due to other factors.

Considering oral corticosteroids are a cornerstone of asthma therapy there are relatively few studies investigating the anti-inflammatory response to these drugs. Bentley *et al* investigated the effects of two weeks of oral corticosteroids on inflammatory changes in the bronchial mucosa in 18 subjects with steroid naïve asthma (8 placebo). There were between six and nine paired biopsies in each group. They demonstrated a significant reduction in eosinophils, CD3 lymphocytes and tryptase positive mast cells in the corticosteroid group [20]. There was a 4.5% improvement in FEV_1 which was not significantly different compared to placebo. There was a significant 1.7 fold improvement in Pc20 compared to placebo. These changes are similar to our study. Djukanovic *et al* undertook a similar study on steroid naïve subjects and found with larger numbers of subjects a significant 10% improvement in FEV1 and a similar magnitude improvement in Pc20 found in the Bentley study [21]. They did not demonstrate any changes in BAL perhaps reflecting relatively mild baseline inflammation in the airway lumen, but there was a significant reduction in CD3 cells, mast cells and

eosinophils in the mucosa. We did not see changes in lymphocytes and mast cells in our study which may reflect differences in baseline corticosteroid treatment. Using an open study design Fukakusa *et al* investigated 13 patients with refractory asthma. They withheld all corticosteroids for one month and then treated with two weeks of 40mg/day of oral prednisolone [22]. Perhaps not surprisingly there was a 35% improvement in $FEV_{1.}$ There was a reduction in CD3 cells, eosinophils and eosinophil related chemokine expression in the bronchial mucosa, but an increase in neutrophils and neutrophil related cytokines. The same group investigated 21 mild/moderate asthmatics taking ICS with documented bronchodilator reversibility and divided them arbitrarily into two groups depending on their response to oral corticosteroids; sensitive if they had a greater than 22% improvement in FEV_1 and resistant if less than 15% improvement [23]. They suggested that the sensitive group had a fall in their mucosal CD3 count (although there was a variable response), and eosinophil count whereas the resistant group did not. In the responders five subjects had an eosinophil count higher than normal (>20 cells per mm2), which in each case normalised with corticosteroids, whereas in the non-responders only two subjects fell outside the normal range, both of whom normalized with corticosteroids, so this would also fit a model in which asthmatics only respond to oral corticosteroids in terms of improvements in lung function if they have active eosinophilic airway inflammation. Wenzel *et al* investigated the immunopathology of severe oral corticosteroid dependent asthma. In contrast to our study they did not find a decrease in airway eosinophils but did find an increase in neutrophils [24]. Lastly Kupczyk *et al* investigated the response to two weeks of oral prednisolone in a large group of severe and mild to moderate asthmatics in a double blind placebo controlled design [25]. They did not undertake bronchoscopy relying on sputum and exhaled nitric oxide (FeNO), as measures of airway inflammation. As in this study they found that improvements in FEV_1 were related to sputum and blood eosinophils with a sputum count of >3% and a FeNO >45ppb providing the best sensitivity. Together these studies are consistent with a model we proposed a number of years ago in which the primary site of action of corticosteroids in asthma is suppression of eosinophilic airway inflammation [26].

In this study all the patients in the severe group were on large doses of ICS and in some cases on oral steroids, yet despite this some still had a significant airway eosinophilia which was corticosteroid responsive. A possible explanation is sub-optimal compliance with inhaled and oral corticosteroids which is very common in asthma and the most frequent reason for asthma being difficult to control [3, 27]. Another possibility is that there was a greater intensity of eosinophilic inflammation requiring higher doses of steroids to control, although one would expect that the steroid receptors would be maximally occupied by the high doses of potent topical steroids these patients were prescribed. A third explanation is that oral steroids were working at a location different from the ICS, either in the periphery of the lung or another organ such as the bone marrow which is somehow driving the inflammatory process.

Corticosteroids increased the numbers of neutrophils present in the airway and blood, an effect that was more marked in airways but not the blood of the severe asthma group for reasons that are not clear. The differences between the mild/moderate and severe groups were modest and not significant but it does suggest an increased sensitivity to the neutrophilic effects of corticosteroids in severe asthma and it is possible therefore that 'neutrophilic' patterns of airway inflammation in some asthmatics is due to a treatment effect rather than the underlying disease process.

A limitation of this study is that it was not placebo controlled. While the staff measuring the inflammatory markers were blinded to the clinical and physiological changes we cannot exclude the possibility that some of the changes observed after oral corticosteroids were due to fluctuations in the disease process independent of the corticosteroids themselves (for example because of regression to the mean). Against this was the very consistent reduction in the blood eosinophil count across all the compartments measured and the requirement for subjects to be recruited during a stable period of disease activity, outside any period of exacerbation. Another weakness is the lack

of any quality of life measures. The two studies used different questionnaires for symptom response and in any case the open nature of the study limits the robustness of changes in patient reported outcomes.

We pooled the results from 2 studies which had followed common protocols. The US study contained 2 independent centres and the UK 1 independent centre. We did not find any consistent differences between the physiological or immunopathology results between the two studies, but interestingly there were differences in the exhaled nitric oxide, sputum, blood and BAL eosinophilia at baseline. The severe group of subjects from the UK had a mean eosinophil count of 3.56 $x10^5$/ml in peripheral blood compared to a count of 1.53 $x10^5$/ml in the USA severe group. This suggests there may have been more Th2 dependent asthma patients in the UK cohort. This may be because the Leicester cohort had a larger number of adult onset subjects (66%) which are often highly eosinophilic compared to the Wake Forest cohort (35%).

In summary our study has shown that severe asthma is characterized by an airway eosinophilia which occurs despite treatment with high doses of ICS. However this is not due to steroid resistance as systemic corticosteroids are effective at reducing peripheral blood eosinophil counts and cytokine concentrations as well as returning the airway eosinophil count to normal, especially in the bronchial lumen. The degree of eosinophilic inflammation at baseline correlated with the degree of improvement in FEV_1 consistent with the idea that corticosteroids work in large part in asthma by inhibiting eosinophilic inflammation. This provides further support for the concept of measuring eosinophilic airway inflammation in order to guide treatment with corticosteroids.

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Conflict of Interest

Ana R Sousa, Richard P Marshall, Roberta Milone and Rick Williamson are GSK employees and shareholders.

Richard Knowles and Peter Chalk were GSK employees at the time this research was carried out.

Linda Warnock was a GSK employee at the time this research was carried out and is currently working as a contractor for GSK.

Reynold Panettieri Jr, Eugene Bleeker, Andrew J Wardlaw, Sarah Bolton, Annette Hastie, Fiona Symon, Beverley Hargadon, Hilary Marshall, Matthew Richardson, Christopher E Brightling, and Pranab Haldar have no relevant competing interests to disclose.

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Table1: Summary of subject demographics by cohort group

	Healthy	**Mild/ Moderate**	**Severe**
No. included in All subjects population, n (%) of total population*	35 (27.0)	32 (24.5)	50 (38.5)
No. of subjects completed to post treatment visit, n (%)	31 (89)	23 (72)	46 (92)
No. of subjects on concurrent medications			
Prednisolone, n (%)		0	12 (24)
LABA, n (%)		2 (6)	8 (16)
Inhaled Steroid plus LABA, n (%)		19 (59)	34 (68)
Montelukast, n (%)		4 (13)	16 (32)
Theophylline, n (%)		0	11 (22)
Mean Age in years (SD)	31.8 (11.3)	36.5 (9.8)	46.1 (11.1)
Age onset (% childhood onset <21 years)			45%
Length of time since asthma diagnosis			22(1-55)
Sex, n (%)			
Female	17 (49)	20 (63)	28 (56)
Male	18 (51)	12 (37)	22 (44)
Mean BMI in kg/m^2 (SD)	25.3 (5.1)	28.4 (5.5)	30.3 (6.9)
Race, n (%)			
White/Caucasian/European	29 (83)	21 (67)	36 (72)
African American/ African	6 (17)	10 (31)	14 (28)
East Asian	–	1 (3)	–
Smokers, N (%)	–	1 (3)	5 (10)
FEV_1 % predicted			
N	33	32	50
Baseline mean (95% CI)	100.4 (96.8, 103.9)	85.3 (80.9, 89.8)	74.7 (69.2, 80.2)
FVC % predicted			
N	13	32	50
Baseline mean (95% CI)	98.8 (94.4, 103.1)	94.0 (90.4, 97.6)	85.3 (80.6, 90.0)
PEFR % predicted			
N	23	28	49
Baseline mean (95% CI)	100.0 (93.8, 106.2)	92.2 (85.7, 98.8)	78.5 (74.1, 83.0)
PC_{20}			
N	13	31	39
Baseline geometric mean (95% CI)	12.8 (8.40, 19.4)	0.76 (0.47, 1.25)	0.81 (0.45, 1.46)

CI: confidence interval; SD: standard deviation
* Total population includes 13 steroid naive intermittent asthmatics (data not presented)

Table 2: Results^ of baseline changes in eosinophils

Eosinophils	**Cohort E: Healthy**	**Cohort B: Mild/ Moderate**	**Cohort C: Severe**	**B vs E ratio**	**C vs E ratio**
Submucosa Counts/mm^2					
N	15	24	38	–	–
Baseline	3.68	4.65	10.78	1.26 (26% increase)	**2.93*** (193% increase)
95% CI	(1.65, 8.20)	(2.44, 8.89)	(6.23, 18.64)	(0.44, 3.62)	(1.15, 7.48)
p-value	–	–	–	0.6578	0.0254
Cell counts Blood absolute (10^5/mL)					
N	35	32	50		
Baseline	1.10	2.41	2.51	**2.19*** (119% increase)	**2.27*** (127% increase)
95% CI	(0.87, 1.40)	(1.87, 3.10)	(2.05, 3.07)	(1.55, 3.07)	(1.66, 3.12)
p-value	–	–	–	<0.0001	<0.0001
Cell counts Sputum (%)					
N	13	20	30	–	–
Baseline	0.68	2.88	3.32	**4.22*** (322% increase)	**4.85*** (385% increase)
95% CI	(0.31, 1.53)	(1.49, 5.60)	(1.92, 5.74)	(1.47, 12.13)	(1.86, 12.66)
p-value	–	–	–	0.0083	0.0017
Cell counts BAL (%)					
N	21	22	43	–	–
Baseline	0.18	0.70	1.04	**3.82*** (282% increase)	**5.67*** (467% increase)
95% CI	(0.10, 0.33)	(0.39, 1.26)	(0.69, 1.58)	(1.66, 8.80)	(2.75, 11.67)
p-value	–	–	–	0.0019	<0.0001

CI: confidence interval; N: number of subjects with baseline measure; BAL: bronchoalveolar lavage; ^ results based on ANOVA with post hoc pairwise comparisons between cohort groups; *significant at the 5% level

Table 3: Results^ of the changes post treatment for pulmonary function tests and PC_{20}..

		Mild/ Moderate	Severe
Pulmonary function tests (% predicted)			
FEV_1	N	23	46
	Baseline	87.8	73.9
	Increase from baseline	2.1	**3.7***
	95% CI of increase	(-1.4, 5.7)	(1.3, 6.2)
	p-value	0.2292	0.0036
FVC	N	23	46
	Baseline	98.5	84.2
	Increase from baseline	-0.6	**3.1***
	95% CI of increase	(-3.6, 2.4)	(1.0, 5.2)
	p-value	0.6783	0.0046
PEFR	N	22	43
	Baseline	93.5	77.2
	Increase from baseline	2.0	**6.0***
	95% CI of increase	(-2.8, 6.7)	(2.6, 9.4)
	p-value	0.4088	0.0007
Methacholine challenge			
PC_{20} (mg/mL)	N	22	27
	Baseline	1.45	0.95
	Ratio from baseline	1.37	1.48
	% increase from baseline	37%	48%
	95% CI of ratio	(0.8, 2.3)	(0.9, 2.3)
	p-value	0.2251	0.0906

CI of increase: confidence interval of difference (difference: post-prednisolone – pre-prednisolone);
CI of ratio: confidence interval of ratio (ratio: post-prednisolone over pre-prednisolone);
N: number of subjects with both pre and post measures;
*significant at the 5% level; ^results based on mixed model ANOVA

Table 4: Results of the changes post treatment for exhaled nitric oxide and cell counts

		Mild/ Moderate	Severe
Exhaled NO	N	15	38
(ppb)	Baseline	26.1	27.5
	Ratio from baseline	**0.65***	**0.67***
	% decrease	**35%**	**33%**
	95% CI of ratio	(0.50, 0.83)	(0.57, 0.78)
	p-value	0.0012	<0.0001
Cell counts Blood absolute (10^5/mL)			
Eosinophils	N	23	45
	Baseline	2.9	2.5
	Ratio from baseline	**0.25***	**0.25***
	% decrease	**75%**	**75%**
	95% CI of ratio	(0.15, 0.41)	(0.18, 0.36)
	p-value	<0.0001	<0.0001
Neutrophils	N	23	45
	Baseline	35.9	43.4
	Ratio from baseline	**1.89***	**1.85***

	% increase	**89%**	**85%**
	95% CI of ratio	(1.64, 2.17)	(1.68, 2.05)
	p-value	<0.0001	<0.0001
Cell counts Sputum (%) with quality control criteria applied			
Eosinophils	N	12	18
	Baseline	3.7	3.6
	Ratio from baseline	**0.35***	**0.22***
	% decrease	**65%**	**78%**
	95% CI of ratio	(0.13, 0.9)	(0.1, 0.47)
	p-value	<0.0001	<0.0001
Neutrophils	N	12	18
	Baseline	38.2	42.5
	Ratio from baseline	1.11	**1.65***
	% increase	11%	**65%**
	95% CI of ratio	(0.62, 1.97)	(1.03, 2.63)
	p-value	0.7196	0.0386
Cell counts BAL (%)			
Eosinophils	N	19	38
	Baseline	0.9	1.0
	Ratio from baseline	**0.36***	**0.28***
	% decrease	**64%**	**72%**
	95% CI of ratio	(0.2, 0.67)	(0.18, 0.42)
	p-value	0.0015	<0.0001
Neutrophils	N	19	38
	Baseline	3.1	2.9
	Ratio from baseline	1.32	**1.77***
	% increase	32%	**77%**
	95% CI of ratio	(0.68, 2.58)	(1.1, 2.85)
	p-value	0.4084	0.0185
Cell counts per mm^2 Submucosa			
Eosinophils	N	18	35
	Baseline	7.6	7.8
	Ratio from baseline	**0.29***	**0.51***
	% decrease	**71%**	**49%**
	95% CI of ratio	(0.12, 0.71)	(0.27, 0.98)
	p-value	0.0075	0.0430
Neutrophils	N	18	31
	Baseline	6.6	10.7
	Ratio from baseline	1.30	1.55
	% increase	30%	55%
	95% CI of ratio	(0.47, 3.55)	(0.72, 3.34)
	p-value	0.6043	0.2554

CI of ratio: confidence interval of ratio (ratio: post-prednisolone over pre-prednisolone); N: number of subjects with both pre and post measures; NO: nitric oxide; BAL: bronchoalveolar lavage;
*significant at the 5% level; ^results based on mixed model ANOVA

Table 5: Results^ of the analyte changes post treatment

		Mild/ Moderate	Severe
Protein markers			
Serum IP-10	N	19	21
(pg/mL)	Baseline	165	193
	Ratio from baseline	**0.52***	**0.61***
	% decrease	**48%**	**39%**
	95% CI of ratio	(0.42, 0.65)	(0.49, 0.75)
	p-value	<0.0001	<0.0001
Serum MDC (pg/mL)	N	19	21
	Baseline	1573	1584
	Ratio from baseline	**0.38***	**0.44***
	% decrease	**62%**	**56%**
	95% CI of ratio	(0.29, 0.5)	(0.34, 0.57)
	p-value	<0.0001	<0.0001
Serum TARC (pg/mL)	N	19	21
	Baseline	66	88
	Ratio from baseline	**0.66***	**0.65***
	% decrease	**34%**	**35%**
	95% CI of ratio	(0.48, 0.9)	(0.48, 0.88)
	p-value	0.0103	0.0063
Serum MCP-1 (pg/mL)	N	19	21
	Baseline	199	162
	Ratio from baseline	**0.55***	**0.74***
	% decrease	**45%**	**26%**
	95% CI of ratio	(0.44, 0.69)	(0.6, 0.92)
	p-value	<0.0001	0.0072
Serum MCP-4 (pg/mL)	N	19	21
	Baseline	70	106
	Ratio from baseline	**0.74***	**0.81***
	% decrease	**26%**	**19%**
	95% CI of ratio	(0.62, 0.89)	(0.69, 0.96)
	p-value	0.0016	0.0072

CI of ratio: confidence interval of ratio (ratio: post-prednisolone over pre-prednisolone);
N: number of subjects with both pre and post measures;
IP-10: interferon-inducible protein-10; TARC: thymus and activation-regulated chemokine; MDC: macrophage-derived chemokine; MCP: monocyte chemotactic protein
*significant at the 5% level; ^results based on mixed model ANOVA