Respiratory Medicine (2010) **104**, 1425–1435

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/rmed

Montelukast added to fluticasone propionate does not alter inflammation or outcomes

Ratko Djukanović [a,*], Susan J. Wilson [a], Wendy C. Moore [b], Steven M. Koenig [c], Michel Laviolette [d], Eugene R. Bleecker [b], W. Bruce Davis [e], Dennis E. Doherty [f], Ronald Olivenstein [g], Elliott Israel [h], Mani S. Kavuru [i], Eric Kleerup [j], Donna S. Reilly [k], Steven W. Yancey [k], Lisa D. Edwards [k], John L. Stauffer [l], Paul M. Dorinsky [m], Nizar N. Jarjour [n]

[a] *University of Southampton, Southampton, UK*
[b] *Wake Forest University School of Medicine, Division of Pulmonary and Critical Care Medicine, Winston-Salem, NC, USA*
[c] *Charlottesville, VA, USA*
[d] *Laval Hospital and University, Quebec, Canada*
[e] *Medical College of Georgia, Augusta, GA, USA*
[f] *University of Kentucky, Lexington, KY, USA*
[g] *Meakins Christie Laboratories, McGill University; Montreal, Canada*
[h] *Brigham and Women's Hospital, Harvard Medical School; Boston, MA, USA*
[i] *East Carolina University, Brody School of Medicine, Division of Pulmonary and Critical Care Medicine; Greenville, NC, USA*
[j] *Pulmonary Medicine Associates Sleep Center, Reno, NV, USA*
[k] *GlaxoSmithKline, Respiratory Medicine Development Center, Research Triangle Park, NC, USA*
[l] *CV Therapeutics, Palo Alto, CA, USA*
[m] *Teva USA, Horsham, PA, USA*
[n] *University of Wisconsin School of Medicine and Public Health, Section of Allergy, Pulmonary and Critical Care, Madison, WI, USA*

Received 20 August 2009; accepted 5 April 2010
Available online 14 August 2010

KEYWORDS
Asthma;
Airway inflammation;
Eosinophils;
Bronchoscopy;
Fluticasone propionate;
Montelukast

Summary
Background: Airway inflammation is a key pathological feature of asthma which underlies its clinical presentation.
Objectives: To examine whether adding a leukotriene modifier to an inhaled corticosteroid produces further clinical and/or anti-inflammatory benefits in patients symptomatic on short-acting β_2-agonists.
Methods: Patients uncontrolled on short-acting β_2-agonists were treated for 12 weeks with either fluticasone propionate (100 mcg BD) or fluticasone propionate (100 mcg BD) and

* Corresponding author. Southampton General Hospital, Tremona Road, Southampton SO16 6YD, United Kingdom. Tel.: +44 2380 794195; fax: +44 2380 777996.
E-mail address: rd1@soton.ac.uk (R. Djukanović).

0954-6111/$ - see front matter
doi:10.1016/j.rmed.2010.04.004

montelukast (10 mg QD) in a randomized, double-blind, parallel group study. Bronchoscopy with endobronchial biopsy and bronchoalveolar lavage (BAL) was performed before and after treatment to compare effects on airway inflammation.
Results: Of 103 subjects enrolled, 89 subjects completed treatment and 82 subjects had matched pair biopsy samples. Submucosal eosinophil counts, the primary endpoint, and asthma control improved to similar extents after both treatments ($p \leq 0.008$). Both treatments significantly reduced submucosal mast cell, CD3+, CD4+, CD8+ and CD25+ cell counts. Submucosal mast cell reduction was greater in the fluticasone propionate plus montelukast group. There were no differences between treatments in BAL markers of inflammation or thickness of sub-epithelial collagen.
Conclusions: Low-dose fluticasone propionate significantly improves clinical disease control and reduces airway inflammation in asthma patients uncontrolled with short-acting β_2-agonists without further improvement when montelukast is added to low-dose fluticasone propionate.

Introduction

Airway inflammation and remodelling are believed to underlie both asthma symptoms and airway hyperresponsiveness, the hallmark pathophysiological abnormality of this disease. For this reason, anti-inflammatory treatment is the cornerstone of all asthma management strategies.[1–3] In view of their broad range of anti-inflammatory effects,[4,5] inhaled corticosteroids (ICS) are recognized as the most effective prophylactic therapy for asthma and are therefore established as first-line treatment.[1–3] However, over the past 20 years, considerable effort has gone into elucidating the roles of individual mediators, including histamine, platelet activating factor, prostaglandins, cysteinyl leukotrienes (CysLTs) and, more recently, cytokines and chemokines. The objective has been to develop therapies that target key mediator pathways, thereby providing better asthma control while allowing reduction of the dose of ICS and reducing side-effects.[6,7] Among the mediators studied, CysLTs have been viewed as possibly being the most important on account of their broad effects, including bronchoconstriction, vasodilatation, mucus secretion and chemotactic action.[8,9] Hence, CysLT antagonists, in particular leukotriene receptor antagtonists (LTRAs), are suggested as having the greatest therapeutic potential, with numerous studies showing positive clinical effects.[10,11] Current guidelines recommend LTRAs as an alternative treatment added to regular ICS treatment[1,2] in patients not adequately controlled with ICS alone. While this results in improved symptom control,[10,11] the mechanisms of this improvement are unknown. LTRAs exert effects on several cells involved in the inflammatory process.[12] Potential effects on eosinophils are possibly the most clinically relevant given the observations that strategies which aim to control asthma symptoms as well as reduce sputum eosinophilia prevent asthma exacerbations more effectively than treatments focused solely on symptom control.[13,14] However, it is not known what additional anti-inflammatory benefits, above and beyond those of ICS, might be provided by LTRAs.

We report here the findings of a large bronchoscopic study. We evaluated whether adding the LTRA, montelukast (MON) 10 mg once daily (QD), to a low dose of the ICS, fluticasone propionate (FP) 100 mcg twice daily (BD), had additional anti-inflammatory effects in patients with mild to moderate asthma inadequately controlled with β_2-agonist therapy alone. Airway eosinophils were chosen as the primary outcome variable of inflammation because they are a hallmark feature of asthma and because of the association between the suppression of airway eosinophilia and better disease control.[14,15]

Methods

Study design and subject characteristics

This randomized, double-blind study (FPD40014) was conducted at 11 centres. Approval of the study was obtained from local ethics committees and all patients provided written informed consent. The study compared the anti-inflammatory and clinical effects of low-dose inhaled FP (100 mcg BID) with MON (10 mg QD) orally (FP+MON) with low-dose inhaled FP alone (FP). Before randomization and after 12 weeks of treatment, endobronchial biopsy and bronchoalveolar lavage (BAL) were undertaken to assess treatment effects on airway inflammation (see Fig. 1). During a 24-day run-in period patients were confirmed as having mild to moderate asthma with symptoms requiring, as indicated by asthma guidelines,[1–3] the introduction of an ICS.

All the patients had asthma for ≥6 months and used only short-acting β_2-agonists for symptom relief for ≥6 weeks before enrollment. Indeed, all of the patients were required not to have used any ICS for at least 6 weeks prior to screening, but the protocol did not allow an ICS washout period prior to randomization. A further requirement was no use of leukotriene modifiers, cromolyn/nedocromil, theophylline products, and anticholinergics or combination products for 6 weeks prior to screening. Study investigators were expected to follow ICH guidelines for the ethical treatment of study subjects, and therefore should not have directed subjects to discontinue ICS (or other asthma medications) for 6 weeks prior study screening. In summary, all patients screened for study entry should have been on their "standard" asthma treatment without recent a recent change in medication. It is possible that some of the

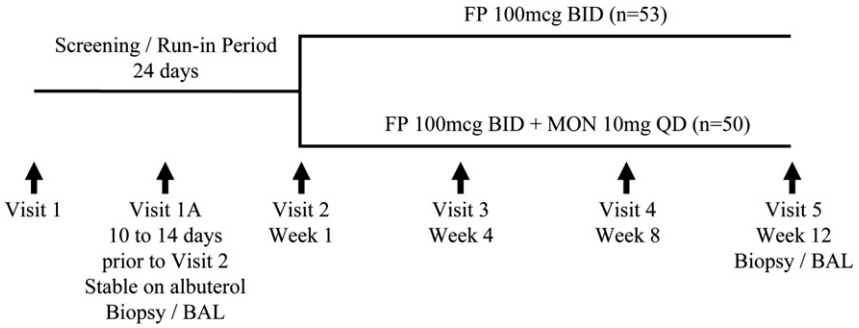

Figure 1 Study design.

patients in this study never used ICS; however, we did not collect data that would provide us with that information. Finally, no use of long-acting or oral beta agonists was a requirement 48 h prior to the screening visit. Clinic charts reflected compliance with these conditions but similarly these data were not collected. After initial screening (Visit 1), any pre-study bronchodilator was replaced with salbutamol two inhalations as needed during the 24-day run-in period. Patients were not restricted in any way in the use of rescue medication. Each patient was instructed to use the study provided short-acting beta agonist for symptom relief; however there was no prohibition against prophylactic use prior to exercise. No defined symptoms were required to be observed to justify use of rescue medication. At Visit 1A (10 to 14 days prior to randomization Visit 2), all subjects met criteria for disease activity as assessed by a composite asthma score on a 0—5 point scale for chest tightness, wheezing, and shortness of breath. Baseline bronchoscopy and BAL were performed at this visit. To ensure safety and tolerability of bronchoscopy at Visit 1A and to proceed to Visit 2 for randomization, subjects were required to have a pre-salbutamol forced expiratory volume in one second (FEV_1) of $>60\%$ of predicted and within $\pm 15\%$ of the highest pre-salbutamol FEV_1 obtained at screening (Visit 1). To fulfill the criteria for treatment with regular ICS, patients had to use salbutamol on ≥ 3 days and/or report a diary card symptom score of >2 on 3 or more days during the week preceding the randomization visit (Visit 2).

Once subjects successfully completed the run-in period and the baseline bronchial biopsy and BAL procedures and met all protocol defined eligibility criteria, they were assigned a unique treatment number as an identification number for blinded study medication. Treatment numbers were assigned consecutively as eligible subjects were randomized. Each site was supplied with open-label FP DISKUS 100 mcg and blinded MON 10 mg capsules and matching placebo. At the randomization visit (Visit 2), eligible subjects were randomized in a 1:1 manner to receive either inhaled FP DISKUS 100 mcg BID plus oral placebo QD or inhaled FP DISKUS 100 mcg BD plus oral MON 10 mg QD.

In order to be randomized at Visit 2, subjects had to fulfill the following criteria: no evidence of respiratory tract infection for ≥ 14 days before Visit 2 and no asthma exacerbation. At Visits 3, 4 and 5 (4 weeks apart), clinical indices of asthma control (peak expiratory flow [PEF], salbutamol use, and symptoms), adverse events and asthma exacerbations were recorded. Subjects who experienced ≤ 2 exacerbations, successfully treated with oral corticosteroids, continued study treatment for 12 weeks and then underwent a second bronchoscopy.

Airway sampling and analysis of inflammation

Endobronchial biopsy and BAL were conducted at all centers using the same protocol after centrally coordinated training. At each bronchoscopy, five or more biopsies were obtained from either the right middle and lower lobes or the lingula and left lower lobe. BAL was performed in the contralateral upper lobe using two 50 mL aliquots of physiological saline. Post-treatment sampling was performed in equivalent segments of the contralateral lung. Biopsies were analysed centrally using monoclonal antibodies for eosinophil cationic protein (ECP), tryptase and T cell surface markers, and measuring the thickness of the subepithelial collagen reported previously described.[16,17]

In order to qualify for detailed immunohistochemical analysis, tissue sections had to contain a minimum of 0.46 mm^2 of submucosal tissue (*lamina propria*), excluding smooth muscle, glands and crush artifact.[18] Immunohistochemical analysis of pre- and post-treatment biopsies for the numbers of eosinophils, total T cells and CD4+ and CD8+ T cell subsets, T cells bearing the interleukin-2 (IL-2) receptor, mast cells and neutrophils was performed as previously described,[16] employing the following monoclonal antibodies: anti-eosinophil cationic protein (EG2; Diagnostic Developments, Uppsala, Sweden) for eosinophils, anti-tryptase antibody (AA1) for mast cells, anti-neutrophil elastase (NP57) for neutrophils, anti-CD3 antibody (UCHT1) for total T cells, MT310 for CD4+ helper cells, DK25 for CD8+ suppressor/cytotoxic T cells and ACT-1 identifying activated T cells bearing the interleukin-2 receptor (IL-2R, CD25; all from DakoCytomation). Positively stained nucleated cells were counted separately in the submucosa and epithelium as previously reported[16] and cell counts expressed per mm^2 of submucosa and per mm length of intact, longitudinally oriented epithelium. In order to assess the effect of treatment on collagen deposition, the thickness of the sub-basement membrane collagen layer, the *lamina reticularis*, was measured underneath the epithelium, also selecting intact, longitudinally orientated epithelium.

Similarly, BAL samples obtained before and after treatment were analysed using standard methods for inflammatory cell counts and mediators including ECP, tryptase, granulocyte macrophage colony stimulating factor (GM-CSF), and interleukin-8 (IL-8).[16–19] Prior to mediator analysis, samples were concentrated by ultrafiltration through a 5 kD cutoff filter.

Interleukin-5 (IL-5), IL-8 and GM-CSF were measured using commercial ELISA kits (R&D Systems, Minneapolis, MN). Tryptase and ECP were measured using the UniCAP system (Pharmacia Diagnostics). BAL concentrations were back-calculated for the degree of ultrafiltration and results expressed as weight per mL of unconcentrated BAL.

Statistical analyses

Data from previous studies[20,21] indicated that 50 subjects per treatment arm would provide ≥74% power to ensure that a 95% confidence interval (CI) for the difference in eosinophils in the submucosa between treatments was contained within the margin of equivalence (25%), where a 50% difference was considered clinically relevant. Analyses of covariance (ANCOVA) adjusting for center and baseline were carried out to construct the appropriate CI for between treatment group difference in each clinical outcome and each biopsy and BAL variable that did not violate the assumption of normality based on Shapiro-Wilk tests. Baseline values were derived from pre-randomization assessments and endpoint values were derived from the final assessments during the double-blind treatment period. For those biopsy and BAL variables determined to be non-normally distributed, differences in treatment group medians and bootstrap confidence intervals were used to summarise treatment differences. The bootstrap confidence intervals were based on one thousand samples (each with replacement) from each treatment group and the 2.5% and 97.5% percentiles of the resulting sampling distribution.[22] Within-treatment group differences were evaluated with paired *t*-tests and Wilcoxon's signed rank tests.

Results

Completed patients

Of 144 patients screened, 103 fulfilled the randomization criteria and underwent baseline bronchoscopy at Visit 1A. The 103 enrolled and randomized subjects were similar between treatment groups at baseline (see Table 1) and all 103 underwent the first bronchoscopy. Eighty-nine subjects completed the study and 88 underwent the second bronchoscopy following treatment ($n = 45$ for FP and $n = 43$ for FP+MON). Fourteen subjects withdrew from the study (see Table 1 and Fig. 2); one subject completed Visit 5 but did not have a second bronchoscopy. Three subjects on FP and four on FP+MON experienced exacerbations (defined as worsening asthma requiring treatment beyond study drug and needing supplemental salbutamol use); each had only one exacerbation, three required oral corticosteroids and none withdrew from the study due to exacerbation.

Immunohistochemical analyses

Eighty-two matched pair biopsies of required quality were analysed for submucosal markers of inflammation (42 for the FP group and 40 for the FP+MON group). Only 25 paired samples (14 in the FP and 11 in the FP+MON group) had sufficiently adequate epithelium for analysis.

Significant within-treatment group reductions from baseline were observed after treatment for submucosal eosinophils, the primary endpoint, for both treatments ($p < 0.001$), with no between-group difference (1.40; 95% CI: −6.61, 8.82) (see Fig. 3). There were also significant ($p \leq 0.010$) within-treatment group reductions in the submucosa for mast cells, neutrophils, and CD3+, CD4+, CD8+ and CD25+ cells (see Table 2). With the exception of mast cells in the submucosa, where a significantly greater reduction was seen in subjects treated with FP+MON, no differences between treatment groups were noted. Both treatments resulted in significant ($p \leq 0.001$), and similar, increases in submucosal neutrophil counts. Significant decreases were also observed for most epithelial inflammatory cells in both groups ($p \leq 0.035$) but with no between-group differences (see Table 3).

The median values for thickness of the *lamina reticularis* changed from 8.21 to 8.49 microns in the FP group, and from 8.49 to 10.62 microns in the FP+MON groups. The change in the thickness of the *lamina reticularis* was significant in the FP treatment group ($p = 0.049$) but not in the FP+MON group, with no significant difference between the two treatment groups (−0.18; 95% CI: −1.97, 0.985). This finding has no simple explanation but is intriguing. There have been many studies demonstrating small changes in RBM thickening after treatment with inhaled steroids or ICS/LABA, but also some studies have shown no change.[23–33] Three months would be considered a short time for changes in RBM, as the positive studies have usually been of longer duration (generally 6–12 months). One could speculate that the change in RBM itself could be due to changes in matrix composition or changes in wall edema (more likely especially if ICS/LABA). While the median increase in the FP+MON group of 0.92 mm ($n = 14$) was significant, the median increase in the FP group was 0.74 ($n = 11$) was not significant and the 95% confidence interval for the difference between these changes contained zero (95% CI: −1.97, 0.99). That is, no significant difference between the median changes was observed. One can conclude that this significant finding is of interest and could be related to treatment, but it must be interpreted cautiously as the sample size was small, the duration of treatment was relatively short, and no adjustments were made for multiple comparisons.

BAL analyses

Paired BAL samples were available for analysis for 86 of the 89 subjects who completed the study. Three had missing cell counts or insufficient BAL recovery. Median relative eosinophil and neutrophil counts (expressed as a percentage of total cells) reduced significantly ($p < 0.04$) only after FP+MON treatment (0.5% to 0.35% for eosinophils

Table 1 Baseline characteristics and change from baseline of patient-recorded clinical data after 12 weeks of treatment.

	FP 100 mcg BID ($n = 53$)	FP 100 mcg BID + MON 10 mg QD ($n = 50$)	Treatment difference (95% CI)[a]
Mean age, years (SD)	29.7 (11.56)	29.4 (8.93)	—
Male/female (%)	36/64	54/46	—
Ethnicity (%)			
Caucasian	85	78	—
Black	8	18	
American Hispanic	6	4	
Asian	2	0	
Withdrawals, *n* (%)	8 (15%)	6 (12%)	—
Adverse event	0	0	
Asthma exacerbation	0	0	
Consent withdrawn	2 (4%)	0	
Lost to follow up	2 (4%)	3 (6%)	
Protocol violation	1 (2%)	1 (2%)	
Other[c]	3 (6%)	2 (4%)	
Morning PEF, L/min (SEM)[b]			
Baseline	413 (13.6)	476 (20.1)	−1.4
Mean change	71.37 (12.85)[d]	70.86 (13.01)[d]	(−39.9, 37.1)
Evening PEF, L/min (SEM)[b]			
Baseline	439 (14.55)	499 (20.32)	3.1
Mean change	54.22 (12.53)[d]	57.05 (11.29)[d]	(−33.8, 40.0)
FEV_1, % predicted (SEM)[b]			
Baseline	76.0 (1.64)	77.43 (1.64)	1.06
Mean change	6.12 (1.32)[d]	6.72 (1.34)[d]	(−2.24, 4.36)
FEV_1, L (SEM)[b]			
Baseline	2.86 (0.09)	3.00 (0.10)	0.03
Mean change	0.24 (0.05)[d]	0.25 (0.05)[d]	(−0.11, 0.16)
FVC, L (SEM)[b]			
Baseline	4.07 (0.12)	4.40 (0.15)	0.01
Mean change	0.13 (0.04)[d]	0.09 (0.06)	(−0.12, 0.14)
FEV_1/FVC, L (SEM)[b]			
Baseline	0.71 (0.01)	0.69 (0.01)	0.00
Mean change	0.04 (0.01)[d]	0.04 (0.01)[d]	(−0.02, 0.02)
Salbutamol use, puffs/24 h (SEM)[b]			
Baseline	2.55 (0.31)	3.17 (0.50)	0.41
Mean change	−2.02 (0.27)[d]	−2.30 (0.55)[d]	(−0.09, 0.92)
Percent rescue-free days (SEM)[b]			
Baseline	28.21 (4.51)	25.42 (5.05)	−4.78
Mean change	46.37(5.38)[d]	44.95 (6.22)[d]	(−17.59, 8.03)

All data are shown mean and standard error of the mean (SEM), except for age (mean and standard deviation [SD]), gender (%), ethnicity (%), and withdrawals (*n* and %).

[a] Estimate of the difference in mean changes from Baseline to Endpoint; 95% CI for the difference in mean changes based on ANCOVA adjusted for center and baseline value.

[b] Analyses based on assumption of normal data.

[c] Other reasons that were reported for randomized subjects withdrawing from the study were: compliance issues ($n = 2$), refused to undergo bronchoscopy at Visit 6 ($n = 1$), insufficient biopsy ($n = 1$), and reported pregnancy ($n = 1$).

[d] Statistically significant ($p \leq 0.008$) within-treatment group change.

and 1.5% to 1.1% for neutrophils), but this reduction was not significantly different when compared with FP treatment (0.7—0.4% for eosinophils and 1.6—1.2% for neutrophils). Lymphocyte and macrophage counts did not change in either group (see Table 4).

The concentrations of IL-8 and GM-CSF were above the detection limits in nearly all pre-treatment samples and did not change in either group after treatment. Pre-treatment BAL concentrations of tryptase, ECP and IL-5 were below the detection limit of the assays performed in 42%, 67%,

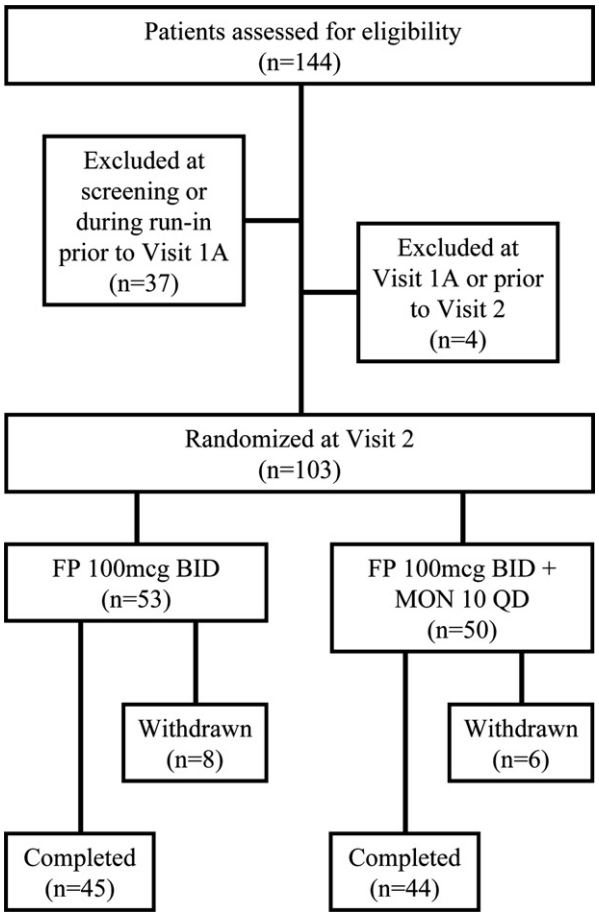

Figure 2 Consort Diagram.

and 87% of the samples, respectively. In view of the small number of evaluable samples for these mediators and cytokines, it was felt that statistical comparison was not appropriate.

Physiological and clinical indices

Within-group changes from baseline for each of the physiologic and clinical indices of asthma control improved significantly and were similar for FP and FP+MON groups (see Table 1). Improvements in morning PEF were observed at the earliest post-randomization time-point (Week 1) in the FP+MON group (36.0 L/min) and in the FP group (29.48 L/min); these improvements continued in each treatment group over the 12-week treatment period. A similar pattern was observed for evening PEF. For the other measures (FEV_1, forced vital capacity [FVC], FEV_1/FVC ratio, 24-h salbutamol use and rescue-free days) there were similar improvements in both groups over the 12 weeks (see Table 1).

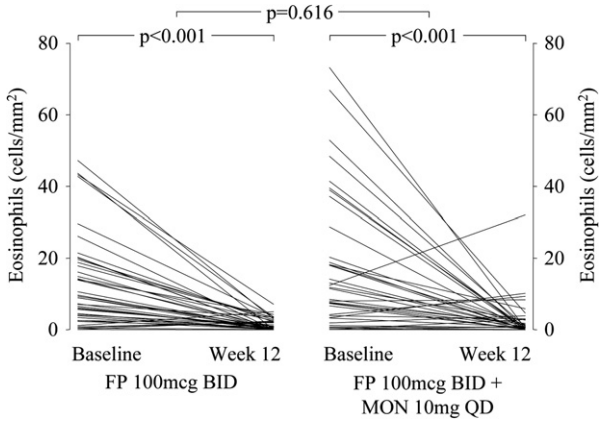

Figure 3 Number of Eosinophils in the Submucosa.

Discussion

This study shows that, in patients requiring first-line anti-inflammatory treatment, adding MON to low-dose FP does not provide greater control of eosinophilic airway inflammation, beyond that seen with FP alone. The study also suggests that MON provides no additional benefit with respect to reducing airway T cells that serve as central orchestrators of allergic inflammation. The only difference between the two treatments in the assessment of inflammatory cell counts or mediators was seen for mast cells where adding MON to FP resulted in a slightly greater reduction in submucosal mast cell counts.

This study is in agreement with that of O'Sullivan et al.,[34] who conducted a crossover study in patients with milder asthma than those in the current study and showed significant, but similar, decreases in eosinophils and mast cells with both treatments. As in the current study, clinical outcomes were similar in patients receiving ICS alone or ICS and MON, which is consistent with findings reported by other investigators.[35–37]

The effects of FP observed in this study are in keeping with other studies demonstrating anti-inflammatory effects of ICS, and the clinical improvements following low-dose ICS in patients with mild to moderate asthma.[6,16,38] However, while asthma symptom control is a central objective in current guidelines, the notion of treating inflammation as well as clinical symptoms has gained prominence recently with evidence that targeting bronchial hyperresponsiveness[39] or sputum eosinophil counts[14] even in patients with well-controlled symptoms may provide further reduction in asthma exacerbations. Furthermore, one of these studies[39] has shown that this approach can affect airway remodelling, as shown by reduced thickness of sub-epithelial collagen. With these considerations in mind, it is justifiable to ask whether more intensive preventative treatment is needed than is currently advised for mild to moderate asthma where symptoms are controlled at levels believed to be acceptable by most asthma guidelines. While a simple solution to this question would be to increase the dose of ICS, this approach would raise concerns about local and systemic side-effects which are dose-dependent.[40] Therefore, combination of an ICS with a non-steroidal drug seems an attractive alternative, particularly, in light of the fact that other studies designed to explore the additive effects, if any, of leukotriene receptor antagonists (e.g. montelukast) combined with ICS when compared to an increased dose of ICS in mild to moderate symptomatic asthma patients, are not conclusive

Table 2 Submucosal counts of mast cells, neutrophils, and CD3+, CD4+, CD8+ and CD25+ T lymphocytes before and after 12 weeks of treatment.

Cell	FP 100 mcg BID		FP 100 mcg BID + MON 10 mg QD		Treatment Difference[c]	95% CI[c]
	Baseline, $n = 51$	Endpoint, $n = 42$	Baseline, $n = 49$	Endpoint, $n = 40$		
Mast cells[a] median (IQR)	22.62 16.36—32.43)	18.44[d] (12.75—23.88)	28.99 (19.35—36.71)	14.11[d] (8.65—21.07)	−9.40	−15.83, −2.96
Neutrophils[b] median (IQR)	21.43 (7.33—53.04)	61.33[e] (22.18—81.55)	21.85 (10.53—41.67)	52.90[e] (29.51—81.23)	1.70	−11.73, 17.14
CD3+ T cells[b] median (IQR)	53.39 (29.56—94.77)	20.68[d] (8.46—42.19)	48.12 (18.66—110.10)	16.74[d] (4.69—27.30)	−15.34	−53.41, 19.80
CD4+ T cells[b] median (IQR)	29.41 (14.89—55.90)	9.40[d] (4.21—17.02)	24.30 (10.14—6.65)	6.30[d] (2.72—12.52)	−2.30	−28.70, 14.14
CD8+ T cells[b] median (IQR)	20.59 (10.38—38.32)	8.12[d] (3.52—16.19)	17.70 (5.92—43.97)	7.96[d] (1.56—12.38)	−2.89	−19.06, 10.34
CD25+ T cells[b] median (IQR)	0.00 (0.00—1.43)	0.00[d] (0.00—0.26)	0.57[d] (0.00—2.87)	0.00[d] (0.00—0.00)	−0.07	−1.63, 0.59

All data (cells/mm^2 of *submucosa*) are shown as median and interquartile range (IQR, 25—75 percentiles).
[a] Analyses based on assumption of normal data.
[b] Analyses based on assumption of non-normal data.
[c] Estimate of the difference in mean or median changes from baseline to endpoint; 95% CI for the difference in mean or median changes.
[d] Statistically significant ($p \leq 0.010$) within-treatment group changes (decreases).
[e] Statistically significant ($p \leq 0.001$) within-treatment group changes (increases).

Table 3 Epithelial counts of mast cells, neutrophils, and CD3+, CD4+, CD8+, CD25+ T lymphocytes before and after 12 weeks of treatment.

Cell	FP 100 mcg BID		FP 100 mcg BID + MON 10 mg QD		Treatment Difference[c]	95% CI[c]
	Baseline, $n = 22$	Endpoint, $n = 29$	Baseline, $n = 21$	Endpoint, $n = 28$		
Mast cells[a] median (IQR)	1.46 (0.75—2.86)	0.00[d] (0.00—0.67)	1.32 (0.62—2.17)	0.00[d] (0.00—0.60)	−0.99	−2.44, 0.46
Neutrophils[b] median (IQR)	0.00 (0.00—1.49)	0.76 (0.00—1.81)	0.00 (0.00—0.00)	0.55 (0.00—3.05)	0.00	−1.64, 3.18
CD3+ T cells[b] median (IQR)	8.05 (2.27—15.85)	0.76[d] (0.00—1.64)	5.65 (1.64—11.73)	0.62[d] (0.00—3.11)	−7.31	−12.99, 5.21
CD4+ T cells[b] median (IQR)	0.82 (0.00—2.50)	0.00[d] (0.00—0.00)	0.00 (0.00—1.74)	0.00[d] (0.00—0.00)	−0.28	−3.64, 2.47
CD8+ T cells$^+$ median (IQR)	3.81 (1.94—9.62)	0.76[d] (0.00—1.83)	5.58 (0.00—8.87)	0.51[d] (0.00—1.42)	−0.45	−7.46, 6.56
CD25+ T cells[b] median (IQR)	0.00 (0.00—0.00)	0.00 (0.00—0.00)	0.00 (0.00—0.00)	0.00 (0.00—0.00)	0.00	0.00, 0.00

All data (cells/mm length of tissue) are shown as median and interquartile range (IQR, 25—75 percentiles).
[a] Analyses based on assumption of normal data.
[b] Analyses based on assumption of non-normal data.
[c] Estimate of the difference in mean or median changes from baseline to endpoint; 95% CI for the difference in mean or median changes.
[d] Statistically significant ($p \leq 0.035$) within-treatment group changes (decreases).

Table 4 Differential cell counts (as percentage of total cells) and concentrations of inflammatory mediators in BAL before and after 12 weeks of treatment.

	FP 100 mcg BID		FP 100 mcg BID + MON 10 mg QD		Treatment difference[a]	95% CI[a]
Cell or mediator	Baseline, $n = 52$	Endpoint, $n = 41$	Baseline, $n = 49$	Endpoint, $n = 38$		
Eosinophils (%)[b] median (IQR)	0.70 (0.20–2.10)	0.40 (0.20–1.40)	0.50 (0.20–1.70)	0.35[c] (0.10–0.80)	−0.05	−0.45, 0.60
	Baseline, $n = 52$	Endpoint, $n = 43$	Baseline, $n = 49$	Endpoint, $n = 41$		
Macrophages (%)[b] median (IQR)	89.50 (85.70–93.70)	90.50 (85.00–95.00)	89.70 (84.40–94.20)	92.10 (87.70–95.50)	0.20	−2.60, 2.60
Lymphocytes (%)[b] median (IQR)	6.40 (4.18–10.00)	6.20 (3.40–9.60)	5.60 (3.00–9.90)	5.70 (2.90–9.60)	0.60	−1.00, 2.20
	Baseline, $n = 52$	Endpoint, $n = 42$	Baseline, $n = 49$	Endpoint, $n = 41$		
Neutrophils (%)[b] median (IQR)	1.60 (0.85–2.30)	1.20 (0.80–2.00)	1.50 (0.60–2.70)	1.10[c] (0.70–2.40)	−0.05	−1.00, 0.90
	Baseline, $n = 52$	Endpoint, $n = 44$	Baseline, $n = 49$	Endpoint, $n = 42$		
GM-CSF (pg/mL)[b] median (IQR)	0.72 (0.32–1.05)	0.40 (0.21–1.02)	0.48 (0.33–0.92)	0.47 (0.28–0.98)	0.08	−0.04, 0.23
IL-8 (pg/mL)[b] median (IQR)	10.75 (6.38–15.04)	10.44 (6.06–14.78)	11.37 (7.60–15.17)	11.29 (7.64–17.91)	−0.47	−4.95, 4.88

Tryptase, IL-5, and ECP cell counts were below detection levels to adequately measure All data are shown as median and interquartile range (IQR, 25–75 percentiles).

[a] Estimate of the difference in median changes from baseline to endpoint; 95% CI for the difference in median changes.

[b] Analyses based on assumption of non-normal data.

[c] Statistically significant ($p \leq 0.036$) within-treatment group changes (decreases).

with respect to improved lung function or decline in airway inflammatory markers.[41–46] Hence, the objective of this study was focused on seeing whether justification for addition of a LT modifier could be found in any residual pathology, e.g. further reduction in eosinophil counts which, many studies have shown, are a good indictator of risk of exacerbation. As shown in Fig. 3, a significant reduction in residual eosinophilia in the airways post-treatment did not demonstrate any benefit from adding MON.

Although the results of our study cannot be extrapolated to more severe disease, where any residual inflammation not adequately controlled by ICS could conceivably benefit from an LTRA, we were unable to show a significant additional anti-inflammatory effect in mild to moderate disease when patients respond well clinically to an ICS alone.

This study was powered to elucidate whether MON has any additional benefits on airway eosinophils. Analysis of other cell types showed a slightly greater reduction in mast cell counts as the only observed additional benefit of MON. The relevance of this finding is unclear, but it could reflect reduced pro-inflammatory effects of mast cell mediators and, conceivably, cytokines which mast cells have been shown to produce.[20] In keeping with observations in numerous *in vivo* and *in vitro* studies of corticosteroids on isolated cells, FP had a pronounced effect on the numbers of T cells which have long been viewed as key orchestrators of inflammation. Inhaled corticosteroids not only reduce the numbers of T cells but also switch off their production of Th2 cytokines.[6,7] To our knowledge, LTRAs have no such effects which might explain the lack of additional effects on inflammation.

The two treatments were not different with respect to effects on the thickness of the *lamina reticularis*. Given that this study showed no benefit from either one of the treatment regimens with respect to collagen deposition and the fact that this is only one of several measures of airways remodelling, longer term studies may be necessary to evaluate the effects of either treatment regimen on remodelling.

This study showed that both FP and FP+MON treatment resulted in a significant rise in neutrophil numbers in the submucosa. The pro-survival effect of corticosteroids on neutrophils *in vitro* and the rise in circulating numbers of neutrophils *in vivo* seen shortly after introduction of these drugs[47] provide a likely explanation for the increase in airway neutrophils. However, the relevance of this finding is unclear and further studies are required to elucidate whether this is associated with increased neutrophil activation.

There are potential limitations in this study. First, ICS act broadly on the inflammatory cascade, including leukotriene pathways by which LTRAs exert their pharmacological effect; these pathways may have been sufficiently controlled by ICS alone. Thus the reduction of numbers of eosinophils, a key source of leukotrienes, could in itself lead to reduced participation of leukotriene pathway products. Corticosteroids also have a direct effect at the cell membrane inhibiting phospholipase A2, which is a precursor of arachidonic acid. This, in turn, modulates the pathways of cyclooxygenase and 5-lipoxygenase. Thus, corticosteroids have an indirect effect on the leukotriene

pathway.On the other hand, 5-lipoxygenase, a key enzyme in this cascade, is located in the nucleus in some cell types and in the cytosol of others. Leukotriene modifiers or anti-leukotrienes constitute 5-lipoxygenase inhibitors and cysteinyl leukotriene receptor antagonists (montelukast). The cysteinyl leukotrienes cause plasma leakage from post-capillary venules and enhance mucus secretion. LD4 and another 5-lipoxygenase derived eicosanoid, 5-oxyo-ETE, are important eosinophil chemoattractants and, subsequently, in inflammation. However, despite the understanding of these mechanisms, the direct role of ICS in the leukotriene pathway is still unclear.[48] Thus, inclusion of a third group of patients treated only with MON may have shown anti-inflammatory effects of this LTRA alone which may have provided further clues as to how this drug works.

Second, in this relatively mild population, an initial positive response to ICS treatment, i.e., reduction in eosinophil counts, was expected. Hence our question was whether addition of another anti-inflammatory drug, i.e. montelukast, could further reduce the cell counts and provide added benefit. Indeed, the vast majority of patients improved with respect to both clinical and pathological outcomes with FP treatment alone, leaving little room for further improvement. Thus a beneficial anti-inflammatory effect of MON could not be excluded in patients in whom the use of an ICS does not result in such a marked anti-inflammatory effect. However, as can be seen in Fig. 3, the reduction in eosinophil counts was marked but not complete. Thus, we can conclude that there is no marked benefit of adding montelukast to fluticasone in this patient population. Third, the concentrations of several relevant mediators were below detection limits in BAL making it difficult to appreciate their relevance in the patients studied. Finally, sampling of mucosal tissue in this study was performed only in the proximal airways accessible by bronchoscopy. Although analysis of BAL, which samples the distal airway and alveolar compartment compartments, showed no differences between the two treatments, it is possible that differential effects might have occurred in the airways mucosa.

In summary, the results of this study show that adding MON to low-dose FP does not provide better control of airway inflammation in patients with asthma requiring first-line anti-inflammatory treatment. Coupled with the lack of additional benefit on measured clinical outcomes, the study suggests that there is little justification for adding MON to regular low-dose FP as first-line preventative therapy for asthma.

Funding

Funding for this research was provided by GlaxoSmithKline.

Conflicts of interest

Declaration of all sources of funding: Dr. Djukanovic reports consultant agreements with and co-founder of Synairgen; research grants from GlaxoSmithKline and Novartis; and has provided legal consultation/expert witness testimony for Synairgen. Dr. Wilson reports research grants from GlaxoSmithKline. Dr. Moore reports contractual lab work for GlaxoSmithKline. Dr. Jarjour reports advisory boards for and honoraria from GlaxoSmithKline and Genentech; and research grants from GlaxoSmithKline, Merck & Co., Genentech, MedImmune, Amgen, University of Nebraska, Roche, Ception Therapeutics, Johnson & Johnson, Curalogic AS, Medicinova, Allergy therapeutics, Wyeth, Greer Laboratories, Inc., and Schering Plough Corporation. Dr. Koenig reports speaker's bureaus for Pfizer, GlaxoSmithKline, and Boehringer-Ingleheim; and research grants from GlaxoSmithKline. Dr. Laviolette reports lectures for Merck-Frost; and research grants from GlaxoSmithKline, Asthma Therapeutics, Merck-Frost, and AstraZeneca. Dr. Bleecker reports consulting and speaking for AstraZeneca; and clinical trials research grants through Wake Forest Health Sciences. Dr. Davis reports no interests to disclose. Dr. Doherty reports research grants from GlaxoSmithKline, Novartis, Schering, Intermune, and Boehringer-Ingleheim. Dr. Olivenstein reports advisory boards and lectures for GlaxoSmithKline, AstraZeneca, and Novartis. Dr. Israel reports advisory boards for Merck, Genentech and Teva; speaker's bureaus for Merck and Genentech; and research grants from Genentech, MedImmune and Johnson & Johnson. Dr. Kavuru reports no interests to disclose. Dr. Kleerup reports honoraria from Ivax/Teva; and research grants from GlaxoSmithKline, BIPI, Roche, Ivax/Teva, MediciNova, Almirall, Novartis, AstraZeneca, Pfizer, Nabi, Chesi, Osiris, Forest, Genentech, and NIH/NHLBI. Ms. Reilly, Mr. Yancey, and Dr. Edwards are employees of GlaxoSmithKline. Drs. Stauffer and Dorinsky were employees of GlaxoSmithKline at the time of the study. Dr. Stauffer is currently an employee of Fibrogen. Dr. Dorinsky is currently an employee of Teva.

Acknowledgements

The authors would like to thank Ibrahim Raphiou, PhD for assistance in preparing the manuscript. The authors also thank Helen Rigden, MSc and Janet Underwood, BSc for their assistance with immunohistochemistry and Lynette Johnson, BS and Annette Hastie, PhD for their assistance with BALF cytokine analyses and cell counts.

References

1. British Thoracic Society. Scottish Intercollegiate Guidelines Network. British guideline on the management of asthma. *Thorax* 2003;**58**(Suppl. 1):1l—94.
2. GINA Report. Global strategy for asthma management and prevention, *From the global strategy for asthma management and prevention*. Global Initiative for Asthma (GINA). Available from: http://www.ginasthma.org; 2006.
3. National Asthma Education and Prevention Program. *Executive summary of the NAEEP expert panel report 3: guidelines for the diagnosis and management of asthma*. Bethesda, MD: National Heart, Lung, and Blood Institute, National Institutes of Health; June 2007. NIH Publication No. 07-4051.
4. Sousa A, Poston R, Lane S, Nakhosteen J, Lee T. Detection of GM-CSF in asthmatic bronchial epithelium and decrease by inhaled corticosteroids. *Am Rev Respir Dis* 1993;**147**:1447—61.
5. Sue-Chu M, Wallin A, Wilson S, Ward J, Sandstrom T, Djukanovic R, et al. Bronchial biopsy study in asthmatics

treated with low and high-dose fluticasone propionate (FP) compared to low-dose FP combined with salmeterol. *Eur Respir J* 1999;**S30**:124.
6. Barnes P. How corticosteroids control inflammation: Quintiles Prize lecture 2005. *Br J Pharmacol* 2006;**148**:245—54.
7. Barnes P. Current issues for establishing inhaled corticosteroids as the anti-inflammatory agents of choice in asthma. *J Allergy Clin Immunol* 1998;**101**:S427—S433.
8. Calhoun W, Lavins B, Minkwitz M, Evans R, Gleich G, Cohn J. Effect of zafirlukast (Accolate) on cellular mediators of inflammation. *Am J Respir Crit Care Med* 1998;**157**:1381—9.
9. Hasday J, Meltzer S, Moore W, Wisniewski P, Hebel J, Lanni C, et al. Anti-inflammatory effects of zileuton in a subpopulation of allergic asthmatics. *Am J Respir Crit Care Med* 2000;**61**: 1229—36.
10. Laviolette M, Malmstrom K, Lu S, Chervinski P, Pujet J, Peszek I, et al. Montelukast added to inhaled beclomethasone in treatment of asthma. *Am J Respir Crit Care Med* 1999;**160**:1862—8.
11. Vaquerizo M, Casan P, Castillo J, Perpina M, Sanchis J, Sobradillo V, et al. Effect of montelukast added to inhaled budesonide on control of mild to moderate asthma. *Thorax* 2003;**58**:204—10.
12. Drazen J, Israel E, O'Byrne P. Treatment of asthma with drugs modifying the leukotriene pathway. *N Engl J Med* 1999;**340**: 197—206.
13. Green R, Brightling C, McKenna S, Hargadon B, Neale N, Parker D, et al. Comparison of asthma treatment given in addition to inhaled corticosteroids on airway inflammation and responsiveness. *Eur Respir J* 2006;**27**:1144—51.
14. Green R, Brightling C, McKenna S, Hargadon B, Parker D, Bradding P, et al. Asthma exacerbations and sputum eosinophil counts: a randomized controlled trial. *Lancet* 2002;**360**: 1715—21.
15. Pizzichini E, Pizzichini M, Leigh R, Djukanovic R, Strek P. Safety of sputum induction. *Eur Respir J* 2002;**20**(Suppl. 37):9s—18s.
16. Jarjour N, Wilson S, Koenig S, Laviolette M, Moore W, Davis W, et al. Control of airway inflammation maintained at a lower steroid dose with 100/50 μg of fluticasone propionate/salmeterol. *J Allergy Clin Immunol* 2006;**118**:44—52.
17. Britten KM, Howarth PH, Roche WR. Immunohistochemistry on resin sections: a comparison of resin embedding techniques for small mucosal biopsies. *Biotech Histochem* 1993;**68**:271—80.
18. Sullivan P, Stephens D, Ansari T, Costello J, Jeffery P. Variation in measurements of basement membrane thickness and inflammatory cell number in bronchial biopsies. *Eur Resp J* 1998;**12**:811—5.
19. Moore W, Hasday J, Meltzer S, Wisnewski P, White B, Bleecker E. Subjects with mild and moderate asthma respond to segmental allergen challenge with similar, reproducible, allergen specific inflammation. *J Allergy Clin Immunol* 2001; **108**:908—14.
20. Kraft M, Martin R, Lazarus S, Fahy J, Boushey H, Lemanske R, et al. Airway tissue mast cells in persistent asthma: predicator of treatment failure when patients discontinue inhaled corticosteroids. *Chest* 2003;**124**:42—50.
21. Chetta A, Foresi A, Del Donno M, Bertorelli G, Pesci A, Oliveri D. Airways remodeling is a distinctive feature of asthma and is related to severity of disease. *Chest* 1997;**111**:852—7.
22. Efron B, Tibshirani R. *An introduction to the bootstrap*. London: Chapman and Hall; 1993.
23. Ward C. Airway inflammation, basement membrane thickening and bronchial hyperresponsiveness in asthma. *Thorax* 2002;**57**: 309—16.
24. Van den Toorn L. Benefit from anti-inflammatory treatment during clinical remission of atopic asthma. *Respir Med* 2005; **99**:779—87.
25. Ward C. Inter-relationships between airway inflammation, reticular basement membrane thickening and bronchial hyper-reactivity to methacholine in asthma; a systematic bronchoalveolar lavage and airway biopsy analysis. *Clin Exp Allergy* 2005;**35**:1565—71.
26. Capraz F. The effect of inhaled budesonide and formoterol on bronchial remodeling and HRCT features in young asthmatics. *Lung* 2007;**185**:89—96.
27. Pavord I. Airway inflammation in patients with asthma with high-fixed or low-fixed plus as-needed budesonide/formoterol. *J Allergy Clin Immunol* 2009;**123**:1083—9.
28. Laitinen A. Tenascin is increased in airway basement membrane of asthmatics and decreased by an inhaled steroid. *Am J Respir Crit Care Med* 1997;**156**:951—8.
29. Hoshino M. Inhaled corticosteroid reduced lamina reticularis of the basement by modulation of insulin-like growth factor (IGF)-I expression in bronchial asthma. *Clin Exp Allergy* 1998;**28**: 568—77.
30. Hoshino M. Inhaled corticosteroids decrease subepithelial collagen deposition by modulation of the balance between matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 expression in asthma. *J Allergy Clin Immuol* 1999;**104**:356—63.
31. Orsida B. Effect of a long-acting β_2-agonist over three months on airway wall vascular remodeling in asthma. *Am J Respir Crit Care Med* 2001;**164**:117—21.
32. Boulet L- P. Airway hyperresponsiveness, inflammation, and subepithelial collagen deposition in recently diagnosed versus long-standing mild asthma. *Am J Respir Crit Care Med* 2000; **162**:1308—13.
33. Olivieri D. Effect of short-term treatment with low dose inhaled fluticasone propionate on airway inflammation and remodeling in mild asthma: a placebo-controlled study. *Am J Respir Crit Care Med* 1997;**155**:1864—71.
34. O'Sullivan S, Akveld M, Burke C, Poulter L. Effects of addition of montelukast to inhaled fluticasone propionate on airway inflammation. *Am J Repir Crit Care Med* 2003;**167**: 745—50.
35. Leigh R, Vethanayagam D, Yoshida M, Watson R, Rerecich T, Inman M, et al. Effects of montelukast and budesonide on airway response and airway inflammation in asthma. *Am J Respir Crit Care Med* 2002;**166**:1212—7.
36. Robinson D, Campbell D, Barnes PJ. Addition of leukotrienes antagonists to therapy in chronic persistent asthma: a randomized double-blind placebo-controlled trial. *Lancet* 2001;**357**:2007—11.
37. Vignola A. Effects of inhaled corticosteroids, leukotriene receptor antagonist, or both, plus long-acting beta$_2$-agonist on asthma pathophysiology: a review of the current evidence. *Drugs* 2003;**63**(Suppl. 2):35—51.
38. Djukanovic R, Wilson J, Britten K, Wilson S, Wall A, Roche W, et al. Effect of an inhaled corticosteroid on airway inflammation and symptoms in asthma. *Am Rev Respir Dis* 1992;**145**: 669—74.
39. Sont J, Willems L, Bel E, van Kreiken J, Vandenbrouke J, Sterke P, et al. Clinical Control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. *Am J Respir Crit Care Med* 1999;**159**:1043—51.
40. Ronald N, Bhalla R, Earis J. The local side effects of inhaled corticosteroids: current understanding and review of the literature. *Chest* 2004;**126**:213—9.
41. Yildrum Z, Ozlu T, Bulbul Y, Bayram H. Addition of montelukast versus double dose of inhaled budesonide in moderate persistent asthma. *Respirology* 2004;**9**:243—8.
42. Perng DW, Huang HY, Lee YC, Perna RP. Leukotriene modifier vs inhaled corticosteroid in mild to moderate asthma: clinical and anti-inflammatory effects. *Chest* 2004;**125**:1693—9.
43. Barnes N, Laviolette M, Allen D, Flood-Page P, et al. Effects of montelukast compared to double dose budesonide on airway

inflammation and asthma control. *Respir Med* 2007;**101**: 1652—8.
44. Ducharme FM. Anti-leukotrienes as add-on therapy to inhaled glucocorticoids in patients with asthma: systematic review of current evidence. *Br Med J* 2002;**324**:1545—8.
45. Ram FS, Cates CJ, Ducharme FM. Long-acting beta2-agonists versus anti-leukotrienes as add-on therapy to inhaled corticosteroids for chronic asthma. *Cochrane Database Syst Rev* 2005; **1**:CD003137.
46. Green RH, Brightling CE, McKenna S, Hargadon B, et al. Comparison of asthma treatment given in addition to inhaled corticosteroids on airway inflammation and responsiveness. *Eur Respir J* 2006;**27**:1144—51.
47. Schleimer R. Glucocorticoids supress inflammation but spare innate immune responses in airway epithelium. *Proc Am Thorc Soc* 2004;**1**:222—30.
48. Funk C, et al. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 2001;**294**:1871—5.