

Original article

Effects of low dose fluticasone/salmeterol combination on surrogate inflammatory markers in moderate persistent asthma

Background: Noninvasive surrogate markers provide valuable information on the asthmatic inflammatory process. We wished to examine the effects of low dose fluticasone/salmeterol combination on different commonly used inflammatory markers in moderate persistent asthma.

Methods: Twenty-five moderate persistent atopic asthmatics were enrolled of whom 20 completed an open label study. Following an initial 4 week steroid washout period in which patients took salmeterol 50 µg dry powder inhaler 1 puff BD, they received the addition of fluticasone as fluticasone 100 µg/salmeterol 50 µg combination dry powder inhaler 1 puff BD for the next 2 weeks.

Exhaled nitric oxide, spirometry, methacholine PD₂₀, sputum/blood eosinophils and sputum/serum eosinophil cationic protein (ECP) were measured following the salmeterol only and fluticasone/salmeterol combination treatment periods.

Results: Compared to salmeterol alone (i.e. after the steroid washout), the use of fluticasone/salmeterol combination conferred significant improvements ($P < 0.05$) in all surrogate markers of inflammation apart from serum ECP. Geometric mean fold changes were 4.3-fold/1.3-fold for sputum/blood eosinophils, 2.2-fold/1.2-fold for sputum/serum ECP, 2.3-fold for methacholine PD₂₀ and 1.8-fold for exhaled nitric oxide.

Conclusions: Surrogate markers apart from serum ECP may be used as a guide to evaluate the anti-inflammatory effects of low dose inhaled corticosteroids.

Sputum markers tend to be more sensitive than blood when assessing the anti-inflammatory response.

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Inflammation of the bronchial mucosa is a characteristic feature of asthma which is thought to result in airway hyperresponsiveness and reversible airflow obstruction. Therefore, to ensure optimum short- and long-term outcomes, suppression of the inflammatory cascade is pivotal in its management. In accordance with current guidelines, inhaled corticosteroids (ICS) are consequently the gold standard first-line therapy in asthma (1, 2).

Assessment of asthma control is conventionally based on a combination of symptoms and tests of airway calibre. However, in many patients these parameters are satisfactorily controlled in the presence of persistent airway inflammation (3, 4), which may lead to remodelling and subsequent fixed airways (5). Sont et al. highlighted the usefulness of monitoring the degree of airway hyperresponsiveness to methacholine as a tool which could guide treatment. In their parallel group study involving 75 patients, it was demonstrated that titrating an inhaled corticosteroid dose according to airway hyperresponsiveness plus symptoms and spirometry resulted in superior control than if the latter alone were used (6).

Invasive measures of determining anti-inflammatory efficacy, such as bronchoalveolar lavage and bronchial biopsy, remain by their nature unsatisfactory methods of examining directly the extent of airway inflammation. This has led to several different noninvasive tools being used to guide clinicians in the optimum treatment of their patients. Surrogate inflammatory markers employed include exhaled nitric oxide (NO), assessment of airway hyperresponsiveness, peripheral blood eosinophils, serum eosinophil cationic protein (ECP) and analysis of the cellular and biochemical constituents of induced sputum.

The purpose of our study was to evaluate the responsiveness of ICS on different surrogate inflammatory markers in moderate persistent asthmatics, as their effects are not fully established in asthmatics of this severity. To facilitate washout of the pre-existing ICS therapy without causing exacerbation, we used salmeterol (SM), which exhibits little or no meaningful *in vivo* anti-inflammatory activity (7–10). We then evaluated the effects of adding low dose fluticasone propionate (FP) to SM as a combination inhaler, in order to coincide with the steep

part of the dose–response curve for corticosteroid anti-inflammatory efficacy. The rationale for this is that low dose FP/SM combination is recommended at step 3 for moderate persistent asthma as an alternative to using a higher dose of ICS alone, and would be realistic therapy in this group of asthmatics.

Materials and methods

Patient selection and design

Atopic asthmatics were enrolled at random from our database of volunteers and attended a screening visit to determine suitability. Moderate persistent asthmatics (between 18 and 65 years) were required to have a forced expiratory volume in 1 s (FEV_1) > 60% of that predicted and demonstrate a provocative dose of methacholine, which caused a 20% fall in FEV_1 (PD_{20}) < 500 µg. All patients were required to be maintained on a beclomethasone dipropionate (BDP) equivalent daily dose of at least 400 µg. In this respect, chlorofluorocarbon (CFC)–BDP equates to budesonide on 1 : 1 µg equivalent basis, hydrofluoroalkane–BDP vs CFC–BDP equates on a 1 : 2 µg equivalent basis and CFC–FP vs CFC–BDP equates on a 1 : 2 µg equivalent basis. Any other second-line controller therapy was discontinued for the duration of the study. All patients entered into the trial had no history of respiratory tract infection and no oral corticosteroid use 3 months prior to screening. Tayside committee on medical research ethics gave approval for the study in which all patients gave written consent.

The study was of an open label design. Eligible patients at screening were required to stop their ICS for 4 weeks as an initial washout period and commence SM 1 puff BD (Serevent Accuhaler 50 µg per actuation, GlaxoSmithKline, Uxbridge, UK) to try and prevent exacerbation. A salbutamol inhaler (Ventolin Accuhaler 200 µg per actuation, GlaxoSmithKline, Uxbridge, UK) was given for as required use. Following the 4-week washout period, they switched to using a FP/SM combination inhaler 1 puff BD (Seretide Accuhaler 100/50, GlaxoSmithKline, Uxbridge, UK) for the following 2 weeks. Criteria for withdrawal from the study included a fall in FEV_1 < 50% predicted, > 8 puffs/day of salbutamol, > 20% fall in usual peak expiratory flow rate or requirement for oral prednisolone or increased dose of ICS based on the judgement of the investigators.

Measurements

Two weeks after commencement of the washout period, patients attended the department for spirometry and symptom assessment; those fulfilling withdrawal criteria discontinued the study. Thereafter, they attended the laboratory after the 4-week washout and 2-week treatment period having withheld salbutamol 6 h prior to attendance and SM or combination inhaler on the morning of their visit (i.e. 12 h since the last dose). On both visits patients had exhaled NO, spirometry, methacholine bronchial challenge and blood taken for eosinophil count and spun 1 h later for subsequent serum ECP measurement. Sputum induction was then performed.

Skin prick testing

All patients required to be atopic to at least one common aeroallergen. On a previous occasion, skin prick testing (Diagenics Ltd.,

Milton Keynes, UK) to eight common aeroallergens (grass, trees, weeds, house dust mite, aspergillus, feathers, dog and cat) had been carried out on the volar aspect of the forearm after antihistamines had been withheld for at least 4 days. Saline (0.9% wt/vol) and histamine solutions (1.7 mg/ml) were used as the negative and positive controls, respectively. A positive response was defined as any wheal with a diameter 3 mm greater than the negative control, 15 min after application.

Nitric oxide

Measurement of exhaled nitric oxide was performed prior to spirometry and bronchial challenge tests. It was measured by a chemiluminescence analyser (Model LR200; Logan Research, Rochester, UK) under standardized conditions (11).

Spirometry

Spirometry was performed according to American Thoracic Society criteria using a Morgan Spiroflow bellows spirometer (PK Morgan Ltd, UK) which was calibrated daily (12).

Methacholine bronchial challenge

The methacholine bronchial challenge was performed using a standardized computer assisted dosimetric method as described by Beach et al. (13). In brief, methacholine was administered in doubling cumulative doses from 3.125 to 3200 µg given at 5-min intervals until a 20% fall in FEV_1 was recorded. The PD_{20} was determined by computer-assisted interpolation from the log dose–response curve.

Sputum induction

Following the methacholine bronchial challenge, sputum induction was carried out using a modification of the method described by Pin et al. (14). Patients were given 400 µg of salbutamol, and once their FEV_1 had recovered to baseline value, inhaled 3, 4.5 and 5% hypertonic saline (10 min each concentration) via an ultrasonic nebulizer (Medix Sonix 2000, Clement Clarke Ltd, Essex, UK) for a maximum of 5 min. After mouth rinsing to reduce salivary contamination, they expectorated at 5 min intervals. Sputum samples were stored on ice and processed within half an hour. Cytospin preparations of dithiothreitol-treated sputum samples were stained using a modified Giemsa stain. Cell counts were performed in a blinded fashion using a $\times 40$ objective. A minimum of 500 nucleated nonsquamous cells were counted in order to quantify the percentage of eosinophils present. Only those cells touching horizontal lines of a graticule grid were counted in order to avoid bias in the cell counts. Slides containing > 20% squamous cells were regarded as representing salivary contamination and were excluded. Each study sample was counted twice in order to minimize intra-observer variability. Sputum supernatant was stored at -70°C for subsequent ECP analysis.

Blood eosinophils and ECP

Peripheral blood eosinophils were measured using a SE9000 haematology analyser (Sysmex UK, Ltd, Bucks, UK) and ECP was measured using a radioimmunoassay kit (Pharmacia, Milton Keynes, UK) with a within assay co-efficient of variation of 10%.

Statistical analysis

The study was powered at 80% to show a between treatment difference of two-fold (i.e. one doubling dose) in methacholine PD₂₀ (the primary endpoint) with a sample size of 16 patients. Analysis of variance was performed to compare postSM washout vs postFP/SM treatment. Apart from spirometry, all data were logarithmically converted to normalize their distribution, and analysed using a ‘Statgraphics’ software package (STSC Publishing Group, Rockville, MD). Data were expressed as geometric mean fold differences.

Results

Patients

Five patients dropped out during the SM washout period due to worsening asthma control. The mean age (SE) of the 20 remaining patients completing the study (at screening) was 40 (3) years. The median steroid dose (as CFC–BDP equivalent dose) was 700 µg (interquartile range 400–1000 µg/day) and the number of positive skin prick tests was 3 (interquartile range 1.75–4). Fourteen patients were using BDP and three were maintained on FP and budesonide. Of these, one patient was using montelukast and two were using salmeterol (all of which were discontinued for the duration of the study). The mean FEV₁ (SE) was 81 (3)% predicted, FEF_{25–75} 53 (4)% predicted and the geometric mean methacholine PD₂₀ (geometric SE) was 59 (15) µg.

Spirometry

Spirometry was measured before and after washout, while all other end points were measured after washout. Serial values for FEV₁ (SE) showed a significant ($P < 0.05$) fall from prewashout: 2.55 (0.15) l to post washout: 2.22 (0.16) l, and subsequently rose significantly after FP/SM: 2.63 (0.72) l. The same significant trend was observed for FEF_{25–75} with respective values being: 2.00 (0.16) l falling to 1.48 (0.14) l and subsequent rise to 1.98 (0.18) l.

Surrogate inflammatory markers

After the 4-week washout period (while maintained on SM alone) only 16 patients underwent a methacholine bronchial challenge as their FEV₁ had fallen to below 60% of that predicted; 15 sets of data were available for sputum analysis as not all patients on both visits could expectorate a sufficient quantity. All surrogate markers of inflammation apart from serum ECP were significantly ($P < 0.05$) improved after treatment (Table 1 and Fig. 1). The fold reduction in eosinophils and ECP was greater in sputum than in blood, while the fold change in methacholine PD₂₀ and NO were of a similar magnitude. The individual data comparing PD₂₀ and sputum eosinophils after SM vs FP/SM combination are shown in Fig. 2.

Table 1. Difference between salmeterol washout and fluticasone/salmeterol combination treatment

	Fold difference (95% CI)
Methacholine PD ₂₀	2.3 (1.4–3.8)*
Exhaled nitric oxide	1.8 (1.4–2.4)*
Blood eosinophils	1.3 (1.1–1.5)*
Serum ECP	1.2 (0.9–1.5)
Sputum ECP	2.2 (1.3–3.9)*
Sputum eosinophils	4.3 (1.8–10.1)*

Absolute values are shown as geometric mean fold differences.

* Denotes significant fold difference ($P < 0.05$).

Discussion

Our results have demonstrated that following a 4-week steroid washout period using SM alone, 20% of patients exacerbated. The subsequent addition of low dose FP as a combination inhaler conferred significant improvements in all surrogate markers of inflammation with the exception of serum ECP. Furthermore, eosinophils and ECP exhibited greater sensitivity in sputum than blood when assessing the anti-inflammatory response. As expected, spirometry fell when stopping ICS and starting SM, and subsequently improved after introduction of FP.

These findings compliment existing literature, as the differential effects of low dose ICS on surrogate inflammatory markers remains to be fully established in asthmatics of moderate severity. For example, Lim et al. evaluated effects of 1600 µg/day of budesonide on a range of inflammatory markers in asthmatics with a well preserved mean FEV₁ (96% of predicted) (15). We chose 200 µg/day of FP as this was a realistic dose in which patients of this severity would be treated and would also coincide with the plateau part of the dose–response curve in terms of airway calibre (16), but would incorporate the steep part for anti-inflammatory activity. Furthermore, the 4-week ICS washout period used in our study allowed comparisons to be made to a suitable baseline in the presence of active inflammation.

Our data demonstrated that the geometric mean improvement in methacholine PD₂₀ amounted to a 2.3-fold increase after ICS treatment. Bronchial challenge tests can be easily performed in the laboratory and outpatient clinic and can often provide useful information on management and response to treatment. Bronchoconstrictor stimuli such as methacholine and histamine act directly upon bronchial smooth muscle causing contraction and airway narrowing. This is in contrast with adenosine monophosphate (AMP) induced bronchoconstriction, which occurs via the degranulation of primed mast cells releasing preformed mediators such as histamine and cysteinyl leukotrienes (17). A study by Van den Berge et al. has suggested that AMP is a more sensitive surrogate of airway inflammation than methacholine (18). Thus, despite the improvement in

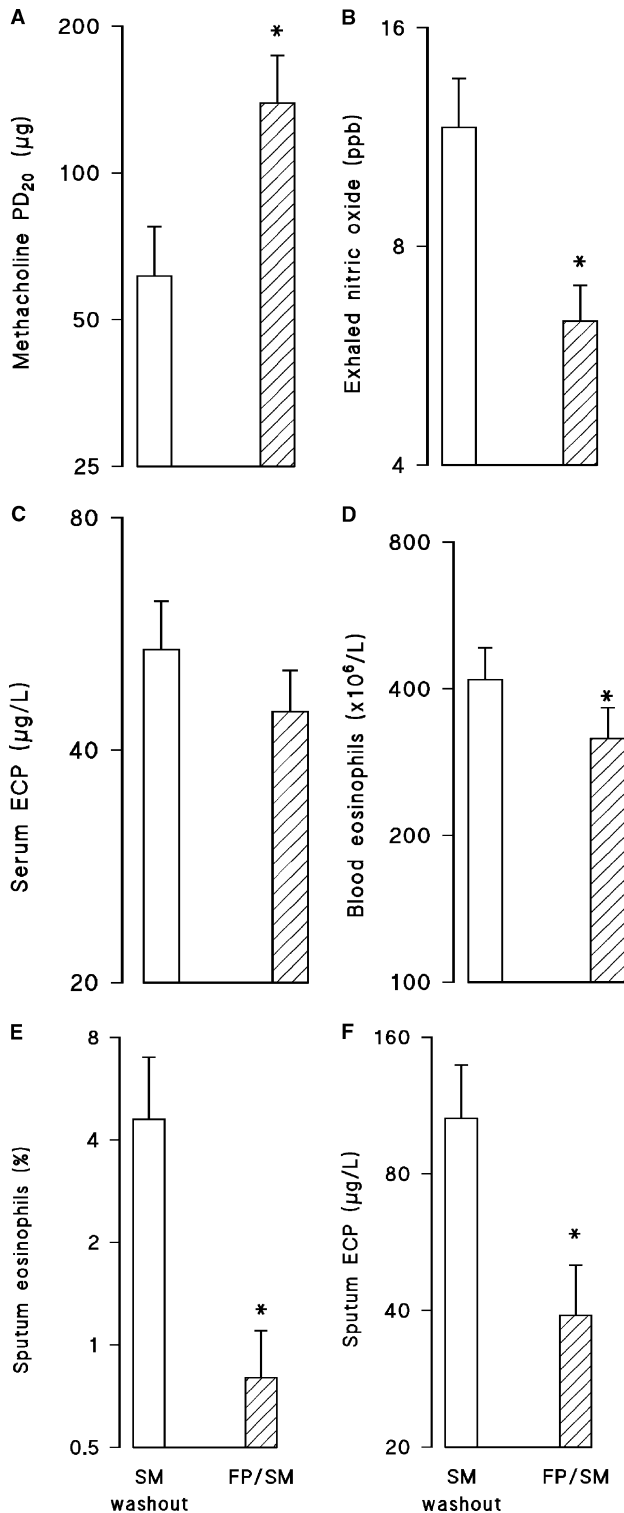


Figure 1. Bar graphs illustrate absolute values after salmeterol washout (empty bars) and after fluticasone/salmeterol treatment (hatched bars). *Denotes significant ($P < 0.05$) difference from salmeterol washout. (A) Methacholine PD₂₀; (B) exhaled NO; (C) serum ECP; (D) blood eosinophils; (E) sputum eosinophils; (F) sputum ECP.

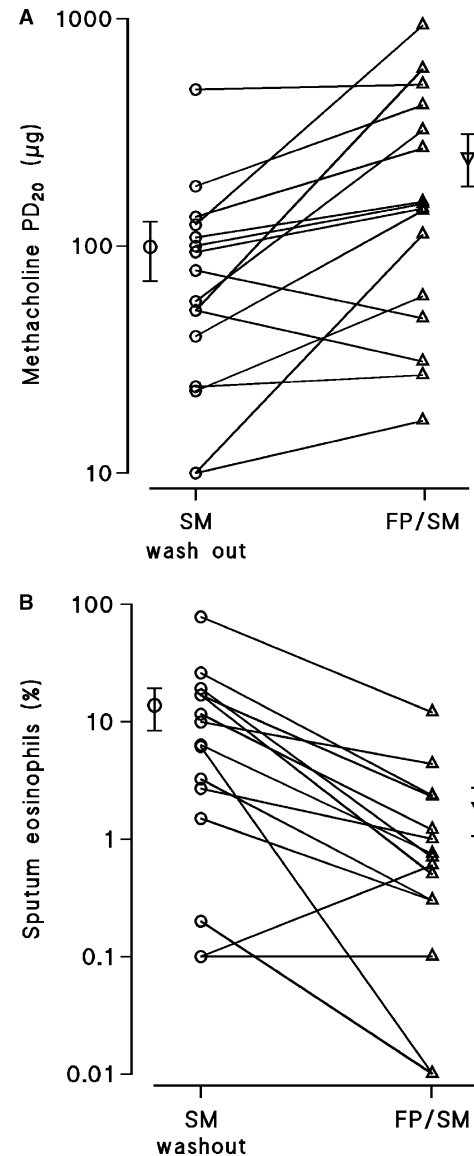


Figure 2. Absolute values for methacholine PD₂₀ ($n = 16$) and sputum eosinophils ($n = 15$) on a log₁₀ scale after salmeterol washout and fluticasone/salmeterol treatment.

methacholine PD₂₀ evident from our study, we may well have observed a greater response if AMP had been employed instead.

NO is produced from respiratory epithelium by an inducible form of NO synthase, while in patients with asthmatic airway inflammation, the expression of NO synthase is increased. Treatment with ICS rapidly reduces levels and serial measurement of exhaled NO may be a useful adjunct in monitoring disease activity (19). Our results show that NO was suppressed to normal levels amounting to a 1.8-fold reduction following treatment with 200 μg/day of FP. This is similar to data using budesonide, which is half as potent as FP, where maximum suppression occurred at 400 μg/day (20, 21).

ECP is a granular protein released from activated eosinophils and therefore considered to be an indirect marker of eosinophil activation. However, similar to our own data, the usefulness of serum ECP as a tool with which to gauge disease activity has led to a relatively inconsistent finding (22, 23). We have demonstrated a significant reduction of sputum but not serum ECP which is in keeping with previous work which failed to show suppression after inhaled budesonide (24). Furthermore, it may be considered that systemic indices of asthma control are more distant indirect markers of inflammatory activity occurring predominantly in the bronchial mucosa.

Sputum induction provides a noninvasive repeatable validated tool in the assessment of airway inflammation (25). Indeed, sputum eosinophils may predict loss of control during steroid tapering (26) and influence management in asthmatics who are difficult to control (27). It is important to point out that in our study patients were asked to expectorate following methacholine bronchial challenge, which has been shown not to alter the biochemical or cellular constituents of sputum (28). Our data demonstrates that sputum markers, especially eosinophils, are particularly sensitive to the effects of ICS and therefore may provide a useful therapeutic adjunct for monitoring disease activity in asthmatics. In a study by Green et al. (29), an asthma management strategy targeted against sputum eosinophils led to significantly fewer severe exacerbations than that using standard guidelines alone. Moreover, the effects on eosinophils and other inflammatory markers were dissociated from lung function and symptoms.

There are some methodological points to discuss about our present study. We proposed that 4 weeks would be a sufficient time length to wash out the effects of preexisting ICS. Moreover, we elected to maintain patients on SM during the washout, to try and prevent severe exacerbations. Since SM has not been shown to exert meaningful anti-inflammatory activity (7–10), we considered that it was a suitable nonsteroidal asthma controller throughout the 4-week period in which ICS were discontinued. However, Jeffery et al. have demonstrated an

anti-neutrophilic effect of SM in mild asthmatics (30). *In vitro* data have demonstrated potential synergy between FP and SM on nuclear glucocorticoid receptor translocation (31), but convincing *in vivo* synergistic effects have not been established. For example, the combination of formoterol and budesonide failed to exhibit potentiation of anti-inflammatory activity compared to budesonide alone (32). Indeed, if we had employed a 4-week comparator cross over a placebo limb, this would have necessitated a further 4-week washout in between treatments. This would have meant patients be kept off ICS therapy for a period of 10 weeks which would be considered unethical. Moreover, even with a 4-week washout, 20% of the patients dropped out due to deteriorating asthma control. However, in a previous study (33) comparing FP/SM combination as an alternative to a higher dose of FP in mild persistent asthmatics, optimizing the inhaled corticosteroid dose with FP conferred superiority compared to half the dose combined with SM. This was in terms of surrogate inflammatory markers but not lung function, of which the latter was superior in the presence of SM. This finding underlines the principle that monitoring lung function alone, without information upon effects of surrogate inflammatory markers, may miss potentially beneficial effects of inhaled corticosteroid therapy.

In conclusion, the results of our study demonstrated that low dose FP/SM combination inhaler compared to SM alone exhibited anti-inflammatory activity across a range of surrogate markers. In addition, suppression of sputum eosinophils and ECP are more sensitive indicators of inflammation than blood.

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Conflicts of interest: the spouse and mother of B.J.L. have stockholdings in GSK, while the Asthma and Allergy Research Group have received support from GSK for attending postgraduate educational meetings and second-hand computer equipment.

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