Blood Eosinophils to Direct Corticosteroid Treatment of Exacerbations of Chronic Obstructive Pulmonary Disease

A Randomized Placebo-Controlled Trial

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***Rationale*: Exacerbations of chronic obstructive pulmonary disease (COPD) and responses to treatment are heterogeneous.**
***Objectives*: Investigate the usefulness of blood eosinophils to direct corticosteroid therapy during exacerbations.**
***Methods*: Subjects with COPD exacerbations were entered into a randomized biomarker-directed double-blind corticosteroid versus standard therapy study. Subjects in the standard arm received prednisolone for 2 weeks, whereas in the biomarker-directed arm, prednisolone or matching placebo was given according to the blood eosinophil count biomarker. Both study groups received antibiotics. Blood eosinophils were measured in the biomarker-directed and standard therapy arms to define biomarker-positive and -negative exacerbations (blood eosinophil count $>$ and $\leq$ 2%, respectively). The primary outcome was to determine noninferiority in health status using the chronic respiratory questionnaire (CRQ) and in the proportion of exacerbations associated with a treatment failure between subjects allocated to the biomarker-directed and standard therapy arms.**
***Measurements and Main Results*: There were 86 and 80 exacerbations in the biomarker-directed and standard treatment groups, respectively. In the biomarker-directed group, 49% of the exacerbations were not treated with prednisolone. CRQ improvement after treatment in the standard and biomarker-directed therapy groups was similar (0.8 vs. 1.1; mean difference, 0.3; 95% confidence interval, 0.0–0.6; $P = 0.05$). There was a greater improvement in CRQ in biomarker-negative exacerbations given placebo compared with those given prednisolone (mean difference, 0.45; 95% confidence interval, 0.01–0.90; $P = 0.04$). In biomarker-negative exacerbations, treatment failures occurred in 15% given prednisolone and 2% of those given placebo ($P = 0.04$).**
***Conclusions*: The peripheral blood eosinophil count is a promising biomarker to direct corticosteroid therapy during COPD exacerbations, but larger studies are required.**
Clinical trial registered with www.controlled-trials.com (ISRCTN 92422949).

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Current guidelines advocate systemic corticosteroids during exacerbations of COPD, but treatment responses are heterogeneous, efficacy is marginal, and the treatment is not without harm. Airway eosinophilia is associated with corticosteroid responsiveness in COPD, and the peripheral blood eosinophil count is a sensitive and specific biomarker for airway eosinophilia during COPD exacerbations.

What This Study Adds to the Field

A biomarker-directed treatment strategy using the peripheral blood eosinophil count to guide corticosteroid prescription can be safely used to treat exacerbations of COPD. Whether this peripheral blood eosinophil biomarker can be used in severe exacerbations requiring hospitalization warrants further investigation.

Keywords: chronic obstructive pulmonary disease; exacerbations; prednisolone; infection; eosinophils

Acute exacerbations of chronic obstructive pulmonary disease (COPD) are associated with substantial morbidity and mortality (1, 2) and are heterogeneous with respect to inflammation (3, 4) and etiology (5–7). Although primarily associated with asthma, eosinophilic airway inflammation is present in some patients with COPD (8). Previous studies have shown that a sputum eosinophilia is associated with a positive response to corticosteroid treatment in stable COPD (9–11), and the sputum eosinophil count can be used to titrate corticosteroid therapy to reduce exacerbations of COPD (12).

Current guidelines advocate the use of systemic corticosteroids during acute exacerbations of COPD because of improvements in the rate of recovery (13, 14); this is despite being associated with significant side effects (15) and with limited benefits in reducing mortality (14). Increased eosinophilic airway inflammation has been shown to occur during exacerbations of COPD, and we have shown that the peripheral blood eosinophil count is a valid biomarker of this pattern of inflammation (16). We hypothesized that the peripheral blood eosinophil count can be used to direct systemic corticosteroid treatment during an exacerbation of COPD

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resulting in reduced total exposure to systemic corticosteroids without adversely affecting the outcome of treatment. To test this hypothesis we undertook a noninferiority study of patients randomized to biomarker-directed corticosteroid therapy versus standard care in patients presenting with an exacerbation of COPD.

METHODS

Participants and Study Design

Subjects with COPD were recruited consecutively from general respiratory clinics at the Glenfield Hospital, Leicester (UK) to enter a randomized biomarker-directed double-blind corticosteroid therapy versus standard care study, wherein the peripheral blood eosinophil count at exacerbation was used to guide corticosteroid treatment in the biomarker-directed arm. At exacerbation, subjects were randomized by minimization (17) for baseline lung function, exacerbation frequency, and sputum eosinophil count and followed up at 2 (post-therapy) and 6 (recovery) weeks after exacerbation (*see* Figure E1 in the online supplement). Randomization and minimization were performed by an independent clinical team. Subjects and study personnel involved in data collection and treatment failure assessment were blinded to randomization, biomarker results, and treatment allocation. Subjects in the biomarker-directed group received a 30-mg prednisolone capsule once daily or identical-appearing placebo for 14 days when the peripheral blood eosinophil count was greater than 2% and less than or equal to 2%, respectively. This cut-off was derived with a high sensitivity aimed to ensure prednisolone treatment in all subjects with a sputum eosinophilia (16). Subjects in the standard group received a 30-mg prednisolone capsule once daily irrespective of the blood eosinophil biomarker results. All subjects received open-labeled broad-spectrum oral antibiotic therapy (amoxicillin, or doxycycline if amoxicillin allergic) for 7 days. Blood eosinophils were measured at exacerbation to define blood eosinophil biomarker-positive and -negative subjects in both study groups (peripheral blood eosinophil levels $\leq$ 2% termed biomarker negative; peripheral blood eosinophil levels $>$ 2% termed biomarker positive), but these results were not disclosed. Exacerbation visits were defined according to the criteria of Anthonisen and colleagues (18) and healthcare use (19), and all subjects were given daily diary cards to complete (20). Data sampling and randomization were only obtained in subjects who were confirmed as having COPD exacerbations and were treatment naive. At all study visits, the following measurements were undertaken: pre- and post-bronchodilator spirometry; health quality questionnaires using the Chronic Respiratory Disease Interviewer-Administered Standardized Questionnaire (CRQ) (21) (McMaster University, Hamilton, Canada); symptom assessment of cough, breathlessness, sputum production, and sputum purulence using the visual analog scale (VAS) (22); blood for measurement of cell differential and C-reactive protein; and sputum for analysis of bacteria, colony-forming units (CFU), virus, and sputum cell differential (23–26). All subjects gave informed written consent, and the study was approved by the local ethics committee and the Medicines and Healthcare Products Regulatory Agency.

Statistical Analysis

Statistical analysis was performed using PRISM version 4 (GraphPad Software, San Diego, CA) and SPSS version 16 (SPSS, Inc., Chicago, IL). Parametric and nonparametric data are presented as mean (SEM) and median (interquartile range), unless stated otherwise. Log-transformed data are presented as geometric mean (95% confidence interval [CI]). The primary objective of the study was to assess whether the blood eosinophil count can be used as a biomarker to direct corticosteroid therapy at the onset of an exacerbation. The primary outcome was to show (*1*) noninferiority in the health status score after treatment between the standard therapy and biomarker-directed therapy study groups; (*2*) equivalence in the proportions of exacerbations associated with a treatment failure defined as the need to start or repeat treatment within 30 days of randomization, hospitalization for any cause, or death, between the standard therapy and biomarker-directed therapy study groups; and (*3*) demonstration of a reduction in corticosteroid therapy prescription in the biomarker-directed therapy study group. To demonstrate noninferiority in health reported outcomes after 14 days of treatment, using the minimally clinical important CRQ mean change of 0.5 (SD, 0.91), 53 subjects were required in each arm to have 80% power at the 5% level. This also provided 95% power at the 5% level to show a 50% reduction in exacerbations requiring corticosteroid therapy, using an exacerbation frequency (SD) of 2.8 (1.7) per year. To exclude a change in the proportion of treatment failure of 20%, from 10 to 30%, between treatment arms, 60 exacerbations in each arm would have a power of 90% at the 5% level. Secondary analysis of health status, symptom scores, lung function, and treatment failures was performed in (*1*) blood eosinophil biomarker-positive and biomarker-negative exacerbations, (*2*) blood eosinophil biomarker-negative exacerbations prescribed prednisolone and placebo, and (*3*) blood eosinophil biomarker-positive and -negative exacerbations prescribed prednisolone. Subjects could only be randomized into the study once, but multiple captured exacerbations were treated as independent events.

Further methodology details are available in the online supplement.

RESULTS

One hundred sixty-four subjects were recruited to enter the study (107 men, 57 women). One hundred nine consecutive subjects with 166 exacerbation events were captured during the study period; 55 and 54 subjects with 86 and 80 exacerbation events, respectively, were randomized to the biomarker-directed and standard therapy arm, as shown in Figure 1. There were 66, 32, 8, and 3 subjects who subsequently had one, two, three, and four captured exacerbations. There were no differences in the clinical characteristics between subjects who were randomized or not (Table E1) or between subjects in the biomarker-directed and standard therapy arm (Table 1). There were 10 severe exacerbations requiring hospitalization. A sputum eosinophil, virus, and bacteria culture positive-associated exacerbation was identified in 17, 32, and 42% of all exacerbations, respectively. There were no differences in the proportions of sputum eosinophil-associated, virus-associated, and bacteria culture positive-associated exacerbations in the biomarker-directed and standard therapy arm at randomization.

Primary Analysis

The primary outcome of noninferiority of health status in the standard therapy and biomarker-directed groups after 2 weeks of treatment was achieved (CRQ mean score change, 0.8 vs. 1.1; mean difference, 0.3; 95% CI, 0.0–0.6; $P = 0.05$; Figure 2a). There was a similar reduction in the CRQ score from baseline to exacerbation in the biomarker-directed and standard therapy arms (0.9 vs. 0.9; mean difference, 0.0; 95% CI, -0.3 to 0.3; $P = 0.97$). There was no difference in FEV_1 or % VAS improvement between biomarker-directed and standard therapy arms after treatment allocation (Figures 2b and 2b). There were 14 treatment failures associated with worsening symptoms of COPD after treatment during the study; 10 occurred in the standard arm and 4 in the biomarker-directed arm, demonstrating at least equivalence with a trend favoring the biomarker-directed arm as there were fewer treatment failures (13 vs. 5%; 95% CI, -1 to 16; $P = 0.07$). In the biomarker-directed group, 49% of the exacerbations were not treated with prednisolone. There were similar proportions of subjects within the standard therapy group and the biomarker-directed therapy group that had one exacerbation (35 vs. 31), two exacerbations (13 vs. 19), three exacerbations (5 vs. 3), and four exacerbations (1 vs. 2).

Secondary Analysis

There were 85 exacerbations that were blood eosinophil biomarker positive given prednisolone, 39 exacerbations that were blood eosinophil biomarker negative given prednisolone, and 42 exacerbations that were blood eosinophil biomarker negative given placebo. Changes in clinical characteristics for biomarker-positive and -negative exacerbations in the biomarker-directed and standard treatment arms at stable, exacerbation, post-therapy, and recovery visits are presented in Table E2.

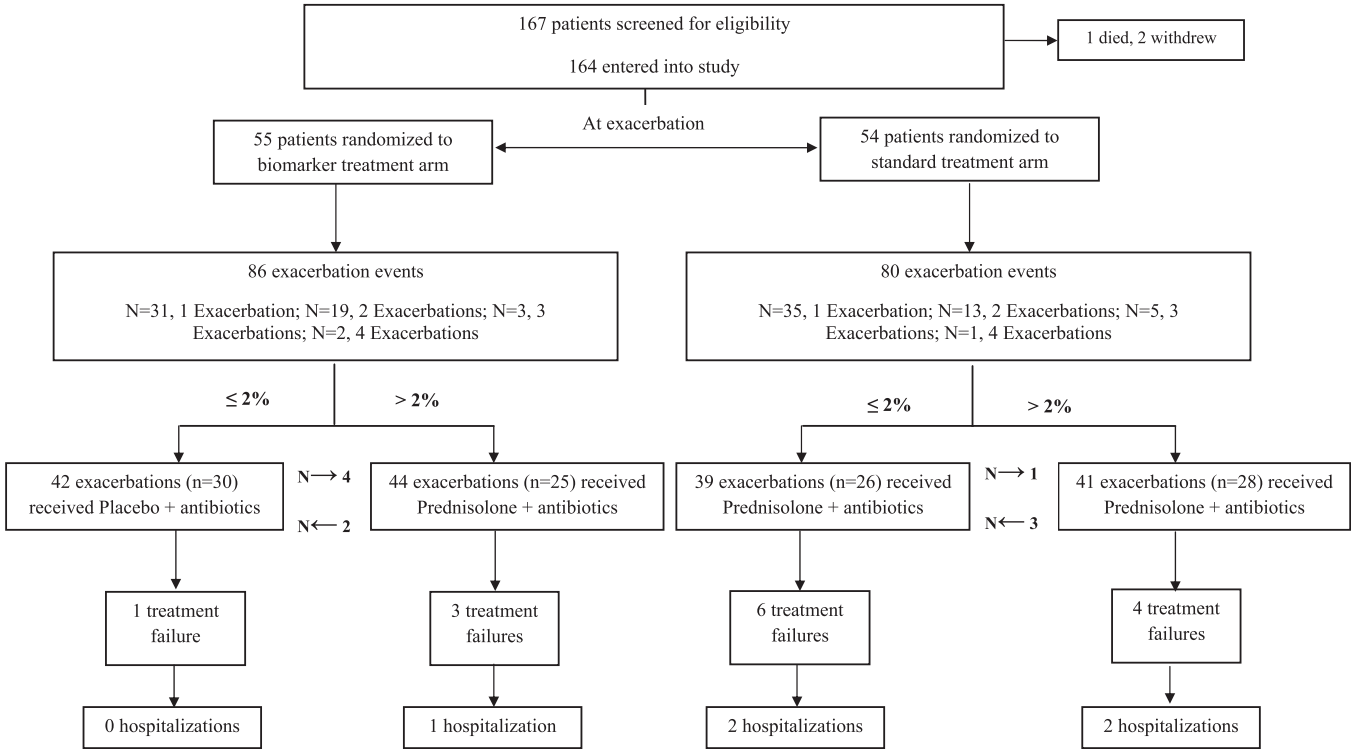

Figure 1. CONSORT diagram for patient enrollment and randomization. Biomarker blood eosinophil levels were measured at exacerbation in both study groups, but only in the biomarker-directed arm were biomarker levels used to direct placebo or matching prednisolone treatment in addition to antibiotic therapy. In the standard arm, all subjects received prednisolone and antibiotic therapy. Four subjects in the biomarker-directed treatment arm switched from placebo to prednisolone treatment, and two subjects switched from prednisolone to placebo. Subjects and study personnel involved in data collection and assessment of treatment failure were blinded to study group allocation, biomarker results, and treatment allocation.

Blood eosinophil biomarker-negative and -positive exacerbations. Baseline and exacerbation health status, lung function, and airway inflammation characteristics in blood eosinophil biomarker-positive and biomarker-negative exacerbations are presented in Table 2. The mean reduction in CRQ from baseline to exacerbation was similar between biomarker-positive and -negative exacerbations (CRQ units, 1.0 vs. 0.9; mean difference, 0.1; 95% CI, −0.2 to 0.3; $P = 0.54$). At exacerbation, blood eosinophil biomarker-negative exacerbations had higher sputum neutrophils, sputum total cell counts, serum CRP, and FEV_1% predicted compared with blood eosinophil biomarker-positive exacerbations (mean [SEM] sputum neutrophils, 86 [2] vs. 78% [3], $P = 0.03$; geometric mean [95% CI] sputum total cell counts $\times 10^6$ cells/g, 9.2 [6.5–13.0] vs. 5.4 [3.9–7.5], $P = 0.03$; median [interquartile range] CRP mg/L, 20 [49] vs. 9 [22], $P < 0.01$; mean [SEM] FEV_1% predicted, 46 [2] vs. 39 [2]; $P = 0.03$). There was a significant difference in absolute and percentage blood eosinophil counts at baseline, exacerbation, post-therapy, and recovery between biomarker-positive and -negative exacerbations (for each visit between groups, $P < 0.01$; Table 3 and Table E2). There were similar proportions of bacteria-associated biomarker-positive and biomarker-negative exacerbations (38 vs. 46%, $P = 0.31$) and virus-associated biomarker-positive and -negative exacerbations (26 vs. 37%, $P = 0.16$). The colony forming units (CFU) at exacerbation were significantly higher in biomarker-negative exacerbations compared with biomarker-positive exacerbations (CFU cells/ml geometric mean [95% CI], 1.1×10^7 [6.2×10^6 to 1.9×10^7] vs. 2.9×10^6 [1.6×10^6 to 5.3×10^6]; $P = 0.002$). A sputum eosinophil–associated exacerbation was found in more biomarker-positive than biomarker-negative exacerbations (31 vs. 2%, $P < 0.001$), whereas only one patient treated with placebo had a sputum eosinophil count (⩾3% nonsquamous cells) at exacerbation. For all exacerbation events captured, the cutoff of 2% blood eosinophil count had a positive predictive value of 91% for identifying a sputum eosinophilia of greater than or equal to 3%.

Blood eosinophil biomarker-negative exacerbations prescribed prednisolone and placebo. Biomarker-negative exacerbations given placebo compared with those given prednisolone had greater improvements in CRQ score after 14 days of treatment (mean change in CRQ [units], 1.01 vs. 0.56; mean difference, 0.45; 95% CI, 0.01–0.90; $P = 0.045$; Figure 3a). There were significantly more treatment failures in subjects with biomarker-negative exacerbations given prednisolone than placebo (15 vs. 2% [95% CI, 1–25], $P = 0.04$). There was no difference in FEV_1 for these groups (Figure 3b). The proportion of exacerbations with no improvement in symptoms after 7 days of treatment was higher in biomarker-negative treated with prednisolone compared with biomarker-negative treated with placebo (21 vs. 4% [95% CI, 0–31], $P = 0.03$). In biomarker-negative exacerbations treated with prednisolone or placebo, there were no differences in the proportions of those associated with bacteria (44 vs. 49%, $P = 0.70$) or virus (36 vs. 38%, $P = 0.87$).

Blood eosinophil biomarker-positive and -negative exacerbations prescribed prednisolone. There was a statistical and clinically significant difference in the CRQ improvement after prednisolone therapy in blood eosinophil biomarker-positive compared with biomarker-negative exacerbations (mean improvement in CRQ [units], 1.11 vs. 0.56; mean difference, 0.56; 95% CI, 0.15–0.96; $P < 0.01$). There was no difference in treatment failure rates between the biomarker-positive and -negative exacerbations treated with prednisolone (8 vs. 15%; 95% CI, −10 to 43; $P = 0.23$). There was a greater recovery over 14 days in biomarker-positive exacerbations treated with prednisolone compared with biomarker-negative exacerbations

TABLE 1. CLINICAL CHARACTERISTICS OF PATIENTS IN RANDOMIZED PLACEBO CONTROLLED-TRIAL

	Biomarker Arm (*N* = 55)	Standard Arm (*N* = 54)	*P* Value*
Male, n (%)	30 (55)	39 (72)	0.07
Age†	70 (49–87)	68 (47–86)	0.27
Current smoker, n (%)	22 (40)	21 (39)	0.91
Ex-smoker, n (%)	32 (58)	32 (59)	0.91
Pack-year history†	52 (10–156)	57 (10–207)	0.47
Exacerbation frequency in previous yr†	3 (1–10)	4 (1–12)	0.12
Body mass index, kg/m^2	27.5 (6.7)	27.3 (5.3)	0.87
Inhaled corticosteroid usage, n (%)	48 (87)	47 (87)	0.97
Inhaled corticosteroid dose, μg‡	1,496 (595)	1,489 (613)	0.96
Atopy, n (%)	13 (24)	7 (14)	0.21
Total IgE, kU/L§	59 (166)	76 (141)	0.66
GOLD I, n, (%)	3 (5.5)	3 (5.6)	0.98
GOLD II, n (%)	23 (41.8)	16 (29.6)	0.18
GOLD III, n (%)	15 (27.3)	15 (27.8)	0.97
GOLD IV, n (%)	14 (25.5)	20 (37.0)	0.38
FEV_1, L‖	1.21 (0.53)	1.18 (0.47)	0.75
FEV_1, %‖	49 (19)	46 (18)	0.29
FEV_1/FVC ratio, %‖	47 (12)	45 (12)	0.35
Reversibility, ml	27 (14)	26 (15)	0.96
Reversibility, %	3.7 (1.2)	3.8 (1.7)	0.95
Sputum total cell count, x 10^6/g¶	2.8 (1.7–4.4)	2.8 (1.9–4.2)	0.93
Sputum neutrophils, %	72 (26)	76 (21)	0.37
Sputum eosinophils, %¶	0.9 (0.6–1.2)	0.8 (0.6–1.2)	0.88
CRQ total, units	3.86 (1.12)	4.14 (1.19)	0.21
VAS total, mm	149 (76)	150 (84)	0.96
Sputum eosinophil–associated exacerbation, %	15	19	0.58
Virus-associated exacerbation, %	32	31	0.95
Bacteria-associated exacerbation, %	44	41	0.22

Definition of abbreviations: CI = confidence interval; CRQ = Chronic Respiratory Disease Questionnaire, scores range between 1 and 7 with higher score representing better health quality; VAS = Visual Analog Scale, performed on 100-mm line from "no symptoms" to "worst symptoms." Higher scores represent worse symptoms (total score addition of measured domains: cough, dyspnea, sputum production, and sputum purulence).

Data presented as mean (SD), unless otherwise stated.

* *t* Test or Mann-Whitney for continuous variables or χ^2 for proportions.

† Mean (range).

§ Median (interquartile range).

‡ Beclomethasone dipropionate equivalent.

‖ Post-bronchodilator.

¶ Geometric mean (95% CI).

treated with prednisolone (area under the % change in VAS curve [95% CI], 516 [449–583] vs. 350 [241–458]; $P < 0.01$) (Figure 3c).

Biomarker phenotype stability. The blood eosinophil biomarker status at baseline had an odds ratio (OR) (95% CI) of 5.5 (2.7–11.0) for predicting the blood eosinophil biomarker status at exacerbation; specifically, blood eosinophil biomarker negative at baseline had an OR of 2.9 (1.6–5.0) for a blood eosinophil biomarker-negative exacerbation, and blood eosinophil biomarker-positive at baseline had an OR 2.2 (1.5–3.2, $P < 0.01$) for a blood eosinophil biomarker-positive exacerbation. A blood eosinophil biomarker-negative status at baseline was identified in 59% of all subjects randomized. In the biomarker-directed group, 80% of patients who were initially assigned prednisolone therapy would have been assigned prednisolone from the baseline blood eosinophil count. Similarly, 59% of patients assigned to placebo at exacerbation would have been assigned this treatment from the baseline blood eosinophil count. In subjects with repeated exacerbation events, comparison of the first and second exacerbation event demonstrated that 22% switched biomarker status (from blood eosinophil biomarker negative to biomarker positive or vice versa), whereas the remainder stayed in the same blood eosinophil biomarker group.

DISCUSSION

In this study we have shown that a biomarker-directed strategy, which used the peripheral blood eosinophil count to guide treatment with corticosteroids, was not associated with an increase in treatment failure or worsening of symptoms compared with standard conventional therapy. More important, we have shown that a biomarker-directed strategy using the peripheral blood eosinophil count can safely reduce prednisolone prescription at exacerbations. There was a trend for outcomes to be better in the group randomized to biomarker-directed treatment versus standard care. Critically, in the subgroup of patients who were blood eosinophil biomarker negative, corticosteroid treatment resulted in worse outcomes compared with placebo. These findings make it very unlikely that we have missed an important difference in outcome in favor of standard, non–biomarker-directed therapy.

A peripheral blood eosinophilia has been previously shown to be associated with an increase in all-cause mortality in patients with airways disease (27–29), and we have previously shown that the peripheral blood eosinophils are a highly sensitive and specific marker of a sputum eosinophilia during exacerbations of COPD (16). It is an attractive biomarker to use in clinical practice as it is simple to measure, widely available at the time of an exacerbation, and reliable. Current guidelines advocate the use of corticosteroids during exacerbations in patients who have increasing symptoms of breathlessness (14). Although studies have shown that corticosteroids can improve lung function and dyspnea scores in the short term (13), these improvements are marginal (30) and need to be weighed against the potential

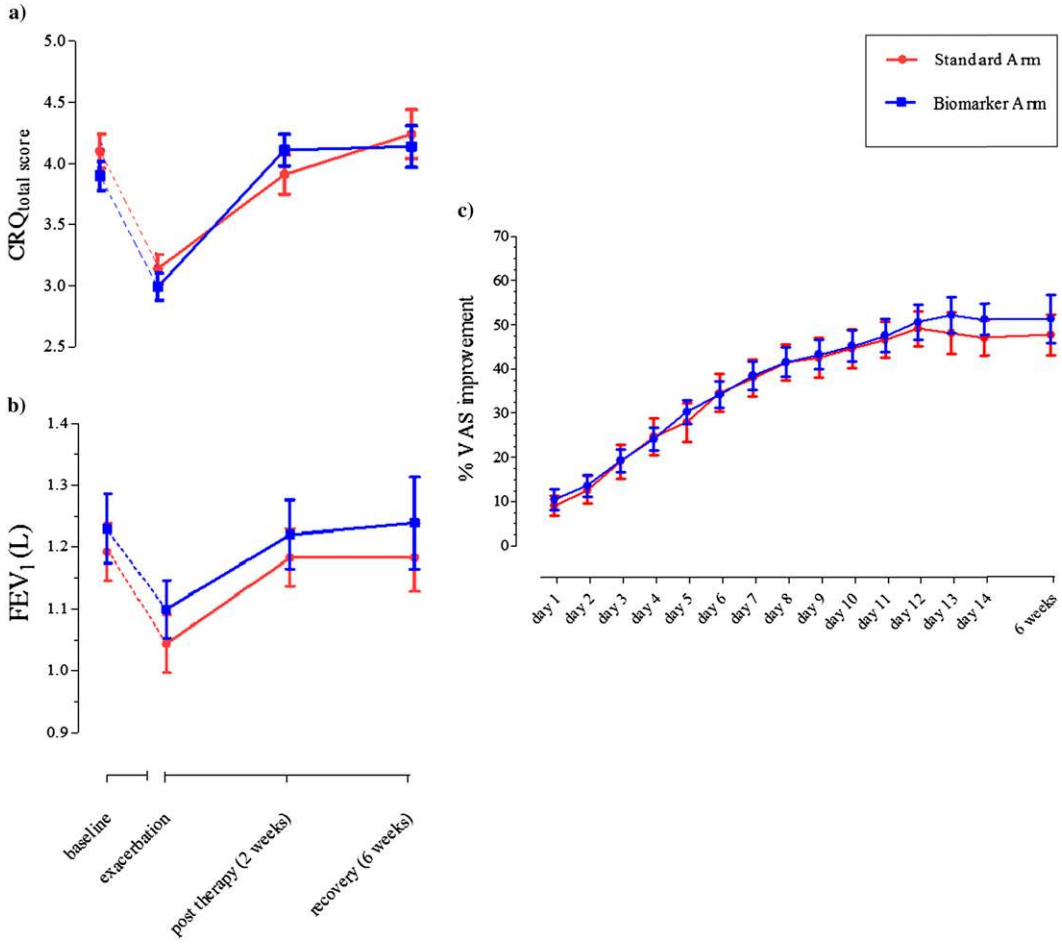

Figure 2. Standard therapy group (*red*) and biomarker-directed therapy group (*blue*). (*a*) Chronic Respiratory Disease Questionnaire total score at baseline, exacerbation, after 14-day placebo or prednisolone treatment (2 wk after exacerbation) and recovery (6 wk after exacerbation) in standard therapy arm (n = 80) and biomarker-directed therapy arm (n = 86). Data presented as mean (SEM). (*b*) FEV_1 at baseline, exacerbation, after 14 days of placebo or prednisolone treatment (2 wk after exacerbation) and recovery (6 wk after exacerbation) in standard therapy arm (n = 80) and biomarker-directed therapy arm (n = 86). (*c*) Percent improvement in visual analog scale total score from exacerbation and for duration of treatment period in exacerbations in standard therapy arm (n = 80) and biomarker-directed therapy arm (n = 86). Data points presented as mean (SEM). CRQ = Chronic Respiratory Disease Questionnaire; VAS = visual analog scale.

for harm in a population who often have significant comorbidities (14, 15). This, together with evidence in stable COPD that patients with eosinophilic airway inflammation respond better to corticosteroid treatment (9–11), provides a strong rationale for a study investigating biomarker-directed therapy. Pooled data analysis has shown that the number needed to harm using corticosteroid therapy in COPD exacerbations is 5, whereas for every 13 patients treated, 1 will develop significant hyperglycemia (14). Our findings suggest that a biomarker-directed strategy for initiating corticosteroid therapy would result in maintenance of the benefits of therapy with a simultaneous reduction in the number harmed by this treatment. Using the peripheral blood eosinophil count as a surrogate marker of eosinophilic airway inflammation, we have shown similar findings of corticosteroid responsiveness in a COPD eosinophilic phenotype but importantly demonstrated this during exacerbations.

TABLE 2. LUNG FUNCTION AND INFLAMMATION AT BASELINE AND EXACERBATION IN ALL EXACERBATIONS CAPTURED CATEGORIZED AS BLOOD EOSINOPHIL BIOMARKER POSITIVE AND BIOMARKER NEGATIVE

	Biomarker Negative ($n = 56$, $n_E = 81$)				Biomarker Positive ($n = 53$, $n_E = 85$)			
	Baseline	Exacerbation	Mean Difference (95% CI)*	*P* Value	Baseline	Exacerbation	Mean Difference (95% CI)*	*P* Value
FEV_1, L†	1.26 (0.56)	1.13 (0.53)	−0.13 (−0.19 to −0.07)	<0.01	1.16 (0.42)	0.99 (0.41)	−0.17 (−0.22 to −0.12)	<0.01
FEV_1, % predicted†	51 (20)	46 (19)	−5 (−7 to −3)	<0.01	46 (18)	39 (18)	−7 (−9 to −5)	<0.01
CRQ score, units	4.00 (1.13)	3.11 (1.05)	−0.88 (−1.06 to −0.70)	<0.01	3.99 (1.20)	3.03 (0.99)	−0.96 (−1.16 to −0.77)	<0.01
Sputum total cell count, $\times 10^6$/g‡	3.0 (2.2–4.0)	8.8 (6.1–12.6)	3.0 (2.0 to 4.3)	<0.01	2.9 (1.9–4.4)	5.6 (3.9–7.9)	2.0 (1.2 to 3.1)	<0.01
Sputum neutrophils, %	72 (22)	85 (20)	12 (6 to 19)	<0.01	80 (20)	80 (22)	0.5 (−7 to 8)	0.90
Sputum eosinophils, %‡	0.7 (0.5–0.9)	0.5 (0.4–0.5)	0.7 (0.5 to 0.9)	<0.01	1.1 (0.8–1.6)	1.7 (1.1–2.6)	1.5 (0.9 to 2.3)	0.09
Blood total cell count, $\times 10^9$ cells/L‡	8.4 (7.8–8.9)	10.3 (9.5–11.1)	1.2 (1.2 to 1.5)	<0.01	9.1 (8.6–9.6)	8.8 (8.3–9.3)	1.0 (0.9 to 1.0)	0.19
Blood neutrophil count, $\times 10^9$ cells/L‡	5.3 (4.9–5.8)	7.3 (6.6–8.1)	1.4 (1.3 to 1.5)	<0.01	5.7 (5.3–6.2)	5.6 (5.2–6.0)	1.0 (0.9 to 1.1)	0.50
Blood eosinophil count, $\times 10^9$ cells/L‡	0.15 (0.13–0.17)	0.11 (0.10–0.13)	0.8 (0.7 to 0.9)	<0.01	0.30 (0.26–0.34)	0.34 (0.31–0.38)	1.2 (1.1 to 1.3)	<0.01
Blood eosinophil %	2.1 (1.4)	1.2 (0.5)	−0.9 (−1.1 to −0.7)	<0.01	3.9 (2.5)	4.4 (2.6)	0.6 (0.0 to 1.1)	0.05
CRP, mg/L	3 (5)	20 (49)	12 (29)	<0.01	5 (10)	9 (22)	0 (13)	0.04

Definition of abbreviations: CI = confidence interval; CRQ = Chronic Respiratory Disease Questionnaire score; CRP = C-reactive protein; n = number of patients; n_E = number of exacerbation events.

Statistical analysis performed using a paired *t* test analysis or Wilcoxon signed rank test. Differences between exacerbation and baseline presented as mean difference (95% CI of difference), fold difference (95% CI of fold difference), and median (interquartile range) of differences as appropriate. Data presented as mean (SD) unless otherwise stated.

*Mean, median, or fold difference as appropriate.

† Post-bronchodilator.

‡ Geometric mean (95% CI).

TABLE 3. LUNG FUNCTION AND INFLAMMATION (ABSOLUTE DATA) AT BASELINE, EXACERBATION, 2 WEEKS AFTER EXACERBATION (POST-THERAPY) AND 6 WEEKS AFTER EXACERBATION (RECOVERY), FOR ALL EXACERBATIONS CATEGORIZED INTO BIOMARKER POSITIVE GIVEN PREDNISOLONE, BIOMARKER NEGATIVE GIVEN PREDNISOLONE, AND BIOMARKER NEGATIVE GIVEN PLACEBO

	Biomarker Positive Given Prednisolone			
	Baseline ($n = 53$)	Exacerbation ($n_E = 85$)	2 wk ($n_E = 85$)	6 wk ($n_E = 41$)
FEV_1, L*	1.16 (0.42)	0.99 (0.41)	1.17 (0.45)	1.19 (0.41)
FEV_1, % predicted*	46 (18)	39 (18)	46 (19)	46 (8)
Sputum total cell count, $\times 10^6$/g†	2.8 (1.9–4.2)	5.4 (3.9–7.5)	2.4 (1.6–3.6)	2.4 (1.7–3.4)
Sputum neutrophils, %	76 (24)	78 (23)	74 (21)	71 (21)
Sputum eosinophils, %†	1.0 (0.8–1.4)	1.6 (1.1–2.3)	0.7 (0.5–0.9)	1.5 (0.9–2.6)
Blood total cell count, $\times 10^9$ cells/L†	9.1 (8.6–9.6)	8.8 (8.3–9.3)	11.6 (10.9–12.4)	9.0 (8.1–9.9)
Blood neutrophil count, $\times 10^9$ cells/L†	5.7 (5.3–6.2)	5.6 (5.2–6.0)	8.1 (7.4–8.9)	5.7 (5.0–6.5)
Blood eosinophil count, $\times 10^9$ cells/L†	0.30 (0.26–0.34)	0.34 (0.31–0.38)	0.19 (0.15–0.23)	0.26 (0.19–0.34)
Blood eosinophil %	3.9 (2.5)	4.5 (2.7)	2.3 (1.9)	3.9 (3.9)
CRP, mg/L‡	5 (10)	9 (22)	3 (9)	3 (6)
	Biomarker Negative Given Prednisolone			
	Baseline ($n = 26$)	Exacerbation ($n_E = 39$)	2 wk ($n_E = 39$)	6 wk ($n_E = 23$)
FEV_1, L*	1.24 (0.49)	1.15 (0.48)	1.22 (0.45)	1.22 (0.43)
FEV_1, % predicted*	48 (20)	44 (19)	48 (19)	46 (17)
Sputum total cell count, $\times 10^6$/g†	2.4 (1.7–3.4)	10.6 (7.0–16.1)	3.8 (2.3–6.3)	2.0 (1.1–3.5)
Sputum neutrophils, %	73 (18)	82 (21)	80 (21)	77 (18)
Sputum eosinophils, %†	0.6 (0.5–0.9)	0.5 (0.4 o 0.6)	0.4 (0.3–0.6)	0.5 (0.3–0.7)
Blood total cell count, $\times 10^9$ cells/L†	9.1 (8.1–10.1)	10.8 (9.8–12.0)	11.9 (10.4–13.7)	8.4 (7.4–9.7)
Blood neutrophil count, $\times 10^9$ cells/L†	5.7 (5.0–6.6)	7.7 (6.8–8.8)	8.2 (7.0–9.7)	5.2 (4.4–6.1)
Blood eosinophil count, $\times 10^9$ cells/L†	0.15 (0.12–0.18)	0.10 (0.09–0.12)	0.11 (0.09–0.14)	0.12 (0.09–0.15)
Blood eosinophil %	2.0 (1.4)	1.1 (0.5)	1.1 (0.8)	1.7 (1.5)
CRP, mg/L‡	5 (8)	18 (42)	10 (20)	6 (10)
	Biomarker Negative Given Placebo			
	Baseline ($n = 30$)	Exacerbation ($n_E = 42$)	2 wk ($n_E = 42$)	6 wk ($n_E = 24$)
FEV_1, L*	1.26 (0.61)	1.10 (0.58)	1.23 (0.58)	1.20 (0.54)
FEV_1, % predicted*	53 (20)	47 (19)	53 (19)	50 (19)
Sputum total cell count, $\times 10^6$/g†	3.5 (2.2–4.4)	8.1 (4.5–10.7)	2.3 (1.4–2.7)	1.7 (0.9–2.0)
Sputum neutrophils, %	72 (25)	88 (17)	78 (18)	77 (19)
Sputum eosinophils, %†	0.7 (0.5–0.9)	0.5 (0.4–0.5)	0.7 (0.5–0.8)	0.8 (0.4–0.9)
Blood total cell count, $\times 10^9$ cells/L†	7.8 (7.3–8.1)	9.7 (8.7–10.2)	8.2 (7.6–8.4)	7.7 (7.0–7.9)
Blood neutrophil count, $\times 10^9$ cells/L†	5.1 (4.6–5.3)	6.9 (6.0–7.4)	5.4 (4.9–5.6)	5.0 (4.5–5.2)
Blood eosinophil count, $\times 10^9$ cells/L†	0.15 (0.12–0.17)	0.12 (0.10–0.13)	0.14 (0.11–0.15)	0.17 (0.13–0.18)
Blood eosinophil %	2.2 (1.5)	1.3 (0.5)	2.0 (1.1)	2.5 (1.5)
CRP, mg/L‡	3 (2)	24 (67)	3 (7)	3 (5)

Definition of abbreviation: CI = confidence interval; CRP = C-reactive protein; n = number of patients; n_E = number of exacerbation events.

Data presented as mean (SD) unless otherwise stated.

* Post-bronchodilator.

† Geometric mean (95% CI).

‡ Median (interquartile range).

We identified that patients who were biomarker positive had higher peripheral blood and sputum eosinophil counts and recovered more quickly with prednisolone than patients who were biomarker negative. In contrast, prednisolone treatment in biomarker-negative patients was associated with more treatment failures and less improvement of health status or symptoms compared with placebo. This finding was unexpected and may have arisen by chance. However, it raises the possibility that the absence of the blood eosinophil biomarker identifies a COPD population whose recovery is adversely affected by corticosteroid therapy, independent of the presence of bacteria or virus at exacerbation. There is increasing evidence that inhaled corticosteroids are associated with an increased risk of pneumonia in COPD (31–33). These findings would suggest that in blood eosinophil biomarker-negative COPD exacerbations, infection may be a primary driver, and thus treatment with corticosteroids is associated with a reduced and possibly detrimental response. We also found that biomarker-positive exacerbations were more likely to have higher blood eosinophils during stable state compared with biomarker-negative exacerbations. Further interrogation of the data also showed that subjects who were biomarker negative at stable state were also more likely to be biomarker negative at the exacerbation event and that repeated exacerbation events remained in the same blood eosinophil biomarker subgroup. Previous work investigating the heterogeneity of COPD exacerbations has shown that the presence of airway eosinophilic inflammation or bacterial pathogen at stable state could predict the exacerbation phenotype (16). In this study, we have determined that a blood eosinophil biomarker status in stable state can predict the exacerbation blood eosinophil biomarker status, highlighting a blood biomarker that has repeatability, has a high predictive value, and is indicative of treatment responsiveness. Whether

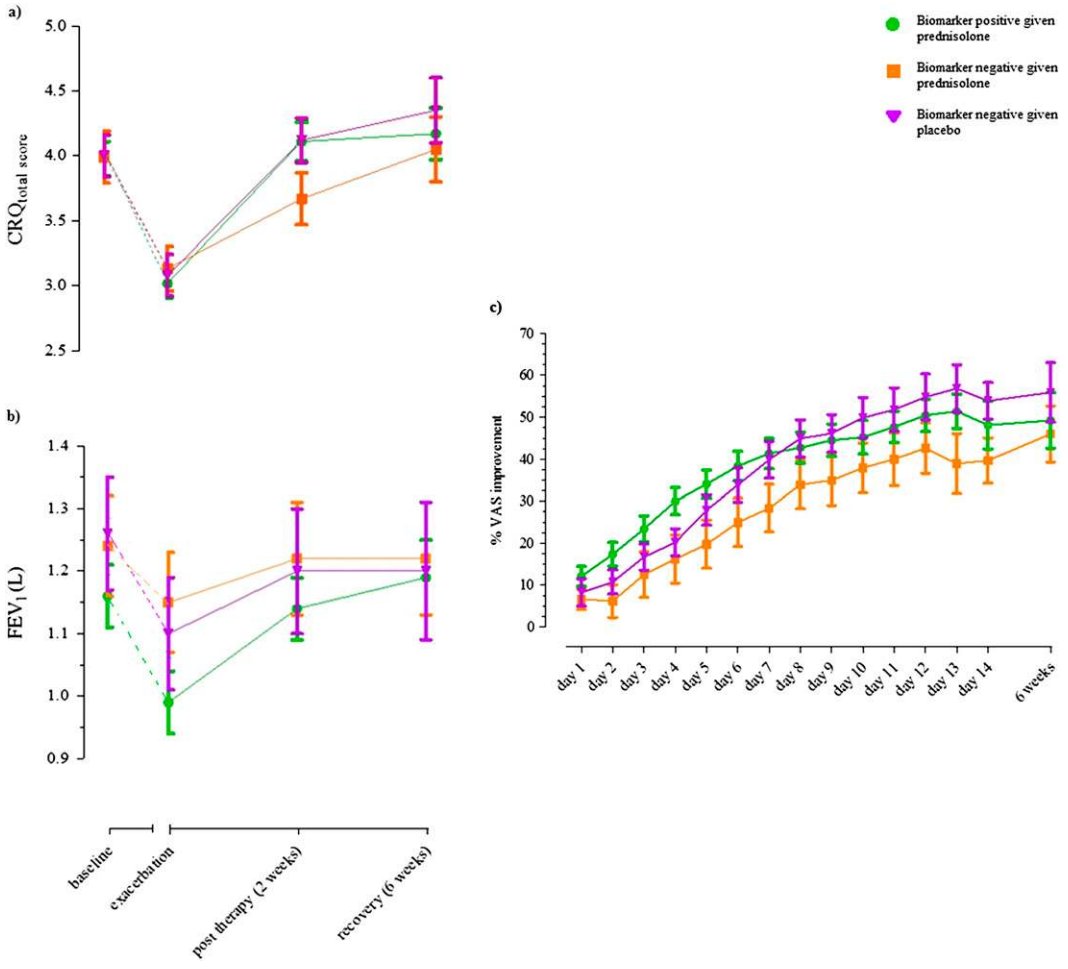

Figure 3. (*a*) Chronic Respiratory Disease Questionnaire total score at baseline, exacerbation, after 14 days of treatment (2 wk after exacerbation) and recovery (6 wk after exacerbation) in exacerbations that were biomarker-positive treated with prednisolone (*green*), biomarker negative treated with prednisolone (*orange*), and biomarker negative treated with placebo (*purple*). Data presented as mean (SEM). (*b*) FEV_1 at baseline, exacerbation, after 14 days of placebo or prednisolone treatment (2 wk after exacerbation) and recovery (6 wk after exacerbation) in exacerbations that were biomarker positive treated with prednisolone (*green*), biomarker negative treated with prednisolone (*orange*), and biomarker negative treated with placebo (*purple*). Data presented as mean (SEM). (*c*) Percent improvement in visual analog scale total score from exacerbation and for duration of treatment period in exacerbations that were biomarker positive treated with prednisolone (*green*), biomarker negative treated with prednisolone (*orange*), and biomarker negative treated with placebo (*purple*). Data points presented as mean (SEM). CRQ = Chronic Respiratory Disease Questionnaire; VAS = visual analog scale.

these patients represent a specific phenotype that can be identified *a priori* and whether baseline knowledge of blood eosinophil biomarker status could direct treatment at the onset of an exacerbation requires further study in larger randomized controlled trials.

A limitation of this study is that the majority of the exacerbations studied were moderate and did not require hospitalization. We would be cautious in extrapolating our findings beyond this group. However, the population we studied reflects a population of patients who exacerbate and present to clinics and primary care, and our findings are likely to be relevant and applicable in this setting (34). Furthermore, our study population had to have a prior history of exacerbations, and therefore they are likely to reflect predominately a frequent exacerbator group. Whether differences in response to therapy exist between infrequent and frequent exacerbator groups requires future study.

Although bacteria are believed to play a role in up to 50% of exacerbations (7), evidence on the benefits of antibiotics is conflicting (35–37). In our study, we have concentrated on targeting corticosteroid therapy and thereby standardized the effects of any bacterial etiology by prescribing open-labeled antibiotic therapy in an aim to eliminate any confounding effects of bacteria within exacerbations. We found no difference in bacteria culture-positive rates in the biomarker-directed and standard therapy arms, so this variable is unlikely to have confounded our comparison between these groups. Treatment failure rates in our study were low, probably reflecting the moderate severity of the exacerbations. It is therefore important that our hypothesis is tested in larger studies including patients hospitalized with severe exacerbations of COPD. These studies should also investigate whether outcomes of biomarker-directed therapy differ by the presence of features such as tapered prednisolone treatment; duration of treatment; and the presence of infection, emphysema, and chronic bronchitis. This study was not powered to study health economic impact of biomarker-directed corticosteroid therapy, and this important potential benefit requires further study. A final concern is that our population may have included patients who had fixed airflow obstruction as a result of asthma and may not be relevant to settings where diagnostic abilities are greater. We acknowledge that this is possible but maintain that we made stringent efforts to reduce a population with characteristics of asthma and were careful to ensure that our population met current diagnostic criteria for COPD (1). It is notable that, as we have shown before (16), features such as atopy and bronchodilator responsiveness were not related to eosinophilic airway inflammation.

In conclusion, a biomarker-directed strategy using the peripheral blood eosinophil count can be used to direct corticosteroid therapy during acute exacerbations of COPD and allows the identification of subgroups that have benefit and detriment from the use of prednisolone treatment. This simple stratification allows for

the identification of clinically important phenotypes of COPD and may identify groups for whom modified therapy is needed. Our data suggest that in the outpatient treatment of exacerbations of COPD, systemic corticosteroids should be only be given to those who have a peripheral blood eosinophil count greater than 2%, but a larger confirmatory study is required. Whether this approach can also be used for patients with severe COPD exacerbations who require hospitalization warrants further investigation.

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References

1. Rabe KF, Hurd S, Anzueto A, Barnes PJ, Buist SA, Calverley P, Fukuchi Y, Jenkins C, Rodriguez-Roisin R, van Weel C, *et al.* Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med* 2007;176:532–555.
2. Halpin D. NICE guidance for COPD. *Thorax* 2004;59:181–182.
3. Bhowmik A, Seemungal TA, Sapsford RJ, Wedzicha JA. Relation of sputum inflammatory markers to symptoms and lung function changes in COPD exacerbations. *Thorax* 2000;55:114–120.
4. Saetta M, Di SA, Maestrelli P, Turato G, Ruggieri MP, Roggeri A, Calcagni P, Mapp CE, Ciaccia A, Fabbri LM. Airway eosinophilia in chronic bronchitis during exacerbations. *Am J Respir Crit Care Med* 1994;150:1646–1652.
5. Papi A, Bellettato CM, Braccioni F, Romagnoli M, Casolari P, Caramori G, Fabbri LM, Johnston SL. Infections and airway inflammation in chronic obstructive pulmonary disease severe exacerbations. *Am J Respir Crit Care Med* 2006;173:1114–1121.
6. Seemungal T, Harper-Owen R, Bhowmik A, Moric I, Sanderson G, Message S, Maccallum P, Meade TW, Jeffries DJ, Johnston SL, *et al.* Respiratory viruses, symptoms, and inflammatory markers in acute exacerbations and stable chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001;164:1618–1623.
7. Sethi S, Murphy TF. Infection in the pathogenesis and course of chronic obstructive pulmonary disease. *N Engl J Med* 2008;359:2355–2365.
8. Saha S, Brightling CE. Eosinophilic airway inflammation in COPD. *Int J Chron Obstruct Pulmon Dis* 2006;1:39–47.
9. Shim C, Stover DE, Williams MH Jr. Response to corticosteroids in chronic bronchitis. *J Allergy Clin Immunol* 1978;62:363–367.
10. Pizzichini E, Pizzichini MM, Gibson P, Parameswaran K, Gleich GJ, Berman L, Dolovich J, Hargreave FE. Sputum eosinophilia predicts benefit from prednisone in smokers with chronic obstructive bronchitis. *Am J Respir Crit Care Med* 1998;158:1511–1517.
11. Brightling CE, Monteiro W, Ward R, Parker D, Morgan MD, Wardlaw AJ, Pavord ID. Sputum eosinophilia and short-term response to prednisolone in chronic obstructive pulmonary disease: a randomised controlled trial. *Lancet* 2000;356:1480–1485.
12. Siva R, Green RH, Brightling CE, Shelley M, Hargadon B, McKenna S, Monteiro W, Berry M, Parker D, Wardlaw AJ, *et al.* Eosinophilic airway inflammation and exacerbations of COPD: a randomised controlled trial. *Eur Respir J* 2007;29:906–913.
13. Davies L, Angus RM, Calverley PM. Oral corticosteroids in patients admitted to hospital with exacerbations of chronic obstructive pulmonary disease: a prospective randomised controlled trial. *Lancet* 1999;354:456–460.
14. Walters JA, Gibson PG, Wood-Baker R, Hannay M, Walters EH. Systemic corticosteroids for acute exacerbations of chronic obstructive pulmonary disease. *Cochrane Database Syst Rev* 2009;CD001288.
15. Niewoehner DE, Erbland ML, Deupree RH, Collins D, Gross NJ, Light RW, Anderson P, Morgan NA. Effect of systemic glucocorticoids on exacerbations of chronic obstructive pulmonary disease. Department of Veterans Affairs Cooperative Study Group. *N Engl J Med* 1999;340:1941–1947.
16. Bafadhel M, McKenna S, Terry S, Mistry V, Reid C, Haldar P, McCormick M, Haldar K, Kebadze T, Duvoix A, *et al.* Acute exacerbations of COPD: identification of biological clusters and their biomarkers. *Am J Respir Crit Care Med* 2011;184:662–671.
17. Treasure T, MacRae KD. Minimisation: the platinum standard for trials? Randomisation doesn't guarantee similarity of groups; minimisation does. *BMJ* 1998;317:362–363.
18. Anthonisen NR, Manfreda J, Warren CP, Hershfield ES, Harding GK, Nelson NA. Antibiotic therapy in exacerbations of chronic obstructive pulmonary disease. *Ann Intern Med* 1987;106:196–204.
19. Rodriguez-Roisin R. Toward a consensus definition for COPD exacerbations. *Chest* 2000;117:398S–401S.
20. Seemungal TA, Donaldson GC, Bhowmik A, Jeffries DJ, Wedzicha JA. Time course and recovery of exacerbations in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2000;161:1608–1613.
21. Guyatt G. Measuring health status in chronic airflow limitation. *Eur Respir J* 1988;1:560–564.
22. Brightling CE, Monterio W, Green RH, Parker D, Morgan MD, Wardlaw AJ, Pavord ID. Induced sputum and other outcome measures in chronic obstructive pulmonary disease: safety and repeatability. *Respir Med* 2001;95:999–1002.
23. Health Protection Agency. Investigation of bronchoalveolar lavage, sputum and associated specimens [Internet]. National Standard Method BSOP 57 Issue 2.3. c2009 [accessed 2010 Aug]. Available from: http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1317132860548
24. Pye A, Stockley RA, Hill SL. Simple method for quantifying viable bacterial numbers in sputum. *J Clin Pathol* 1995;48:719–724.
25. Bisgaard H, Zielen S, Garcia-Garcia ML, Johnston SL, Gilles L, Menten J, Tozzi CA, Polos P. Montelukast reduces asthma exacerbations in 2- to 5-year-old children with intermittent asthma. *Am J Respir Crit Care Med* 2005;171:315–322.
26. Pizzichini MM, Popov TA, Efthimiadis A, Hussack P, Evans S, Pizzichini E, Dolovich J, Hargreave FE. Spontaneous and induced sputum to measure indices of airway inflammation in asthma. *Am J Respir Crit Care Med* 1996;154:866–869.
27. Hospers JJ, Schouten JP, Weiss ST, Postma DS, Rijcken B. Eosinophilia is associated with increased all-cause mortality after a follow-up of 30 years in a general population sample. *Epidemiology* 2000;11:261–268.
28. Hospers JJ, Schouten JP, Weiss ST, Rijcken B, Postma DS. Asthma attacks with eosinophilia predict mortality from chronic obstructive pulmonary disease in a general population sample. *Am J Respir Crit Care Med* 1999;160:1869–1874.
29. Hospers JJ, Rijcken B, Schouten JP, Postma DS, Weiss ST. Eosinophilia and positive skin tests predict cardiovascular mortality in a general population sample followed for 30 years. *Am J Epidemiol* 1999;150:482–491.
30. Aaron SD, Vandemheen KL, Hebert P, Dales R, Stiell IG, Ahuja J, Dickinson G, Brison R, Rowe BH, Dreyer J, *et al.* Outpatient oral prednisone after emergency treatment of chronic obstructive pulmonary disease. *N Engl J Med* 2003;348:2618–2625.
31. Calverley PM, Anderson JA, Celli B, Ferguson GT, Jenkins C, Jones PW, Yates JC, Vestbo J. Salmeterol and fluticasone propionate and survival in chronic obstructive pulmonary disease. *N Engl J Med* 2007;356:775–789.
32. Ernst P, Gonzalez AV, Brassard P, Suissa S. Inhaled corticosteroid use in chronic obstructive pulmonary disease and the risk of hospitalization for pneumonia. *Am J Respir Crit Care Med* 2007;176:162–166.
33. Kardos P, Wencker M, Glaab T, Vogelmeier C. Impact of salmeterol/fluticasone propionate versus salmeterol on exacerbations in severe chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2007;175:144–149.
34. National Clinical Guideline Centre. Chronic obstructive pulmonary disease: management of chronic obstructive pulmonary disease in adults in primary and secondary care [Internet]. London: National Clinical Guideline Centre; c2010 [accessed 2010 Aug]. Available from: http://guidance.nice.org.uk/CG101/NICEGuidance/pdf/English
35. Rothberg MB, Pekow PS, Lahti M, Brody O, Skiest DJ, Lindenauer PK. Antibiotic therapy and treatment failure in patients hospitalized for acute exacerbations of chronic obstructive pulmonary disease. *JAMA* 2010;303:2035–2042.
36. Puhan MA, Vollenweider D, Steurer J, Bossuyt PM, Ter RG. Where is the supporting evidence for treating mild to moderate chronic obstructive pulmonary disease exacerbations with antibiotics? A systematic review. *BMC Med* 2008;6:28.
37. Sethi S. The problems of meta-analysis for antibiotic treatment of chronic obstructive pulmonary disease, a heterogeneous disease: a commentary on Puhan *et al. BMC Med* 2008;6:29.