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Inhaled corticosteroid dose-response in asthma: should we measure inflammation?

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Keywords

Asthma; inhaled corticosteroid; dose-response; inflammation; symptoms; spirometry; FeNO; airway hyper-responsiveness; ECP; blood eosinophils.

Abbreviations

AHR Airway hyper-responsiveness

ANOVA Analysis of variance

23	AQLQ	Asthma Quality of Life Questionnaire
24	ATS	American Thoracic Society
25	BDP	Beclomethasone Dipropionate
26	BTS	British Thoracic Society
27	Bud	Budesonide
28	CFC	Chlorofluorocarbon
29	CI	Confidence interval
30	CIC	Ciclesonide
31	DD	Doubling dilution
32	DDD	Doubling dilution difference
33	ECP	Eosinophilic cationic protein
34	Eos	Eosinophils
35	FEF ₂₅₋₇₅	Forced expiratory flow between 25-75% of forced vital capacity
36	FeNO	Fractional exhaled nitric oxide
37	FEV ₁	Forced expiratory volume in 1 second
38	FP	Fluticasone propionate
39	GMFD	Geometric mean fold difference
40	HFA	Hydrofluoroalkane
41	Hist PC ₂₀	Provocative concentration of histamine causing 20% fall in FEV ₁
42	ICS	Inhaled corticosteroid
43	LABA	Long-acting beta-2 agonist
44	Mann PD ₁₀	Provocative dose of mannitol causing a 10% fall in FEV ₁

45	Mann PD ₁₅	Provocative dose of mannitol causing a 15% fall in FEV ₁
46	MCID	Minimum clinically important difference
47	Meth PC ₂₀	Provocative concentration of methacholine causing 20% FEV ₁ fall
48	%Pred	Percentage of predicted
49	Ppb	Parts per billion
50	RCT	Randomised controlled trial

51

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53

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56

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60

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62

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64

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66

1 **Introduction**

2

3 Asthma is a heterogeneous chronic inflammatory disease of global importance¹,
4 which places a significant burden both on individual patients and on health care
5 services, where many patients remain inadequately treated^{2,3} with an ongoing
6 attendant mortality⁴. The concept of achieving ‘total asthma control’⁵ is
7 important for reducing the future risk of exacerbations⁶⁻⁸. It is therefore
8 imperative that we have robust procedures for accurate diagnosis, measurement
9 of severity, prediction of future risk, along with appropriate personalised
10 treatments to achieve this goal. Nevertheless, present guidelines for the
11 identification and treatment of asthma merely include symptoms and lung
12 function measurements^{5,9}. The Royal College of Physicians’ recent National
13 Review of Asthma Deaths report⁴ found that only 39% of patients who died were
14 actually labelled as having ‘severe’ asthma according to current guidelines, with
15 the remainder therefore labelled as ‘mild’ or ‘moderate’, suggesting we may not
16 be accurately identifying those at greatest risk.

17

18 Measurement of inflammatory outcomes has improved our understanding of
19 asthma, evolving personalised treatment. Studies have shown that titrating
20 steroid therapy against inflammation may improve outcomes such as
21 exacerbation rates¹⁰⁻¹². For example in one primary care based study titrating
22 inhaled corticosteroid dose against mannitol challenge verses a reference
23 strategy, resulted in a 27% significant reduction in mild exacerbations but no
24 difference in severe exacerbations¹¹. Similar findings were observed in another
25 study using methacholine challenge¹³. Green et al. demonstrated this by titrating

steroid treatment against sputum eosinophil counts, resulting in significantly fewer severe exacerbations compared to standard guideline driven treatment¹⁰. It is interesting that this was achieved with no difference in overall mean dosage of ICS between the two groups, suggesting that for the individual, any steroid titration was performed at the right time for them when their levels of inflammation were greater. However, other studies have suggested a more muted response to inflammatory steroid titration in unselected asthmatic patients^{14, 15}.

Price et al. demonstrated retrospectively, in a primary care cohort, that asthmatic patients with higher blood eosinophil counts fared worse in terms of experiencing more severe exacerbations and poorer asthma control¹⁶. Moreover, eosinophilic inflammation may be masked when using a long-acting beta-2 agonist (LABA) as a steroid-sparing agent^{17, 18}. Sputum and blood eosinophilia in asthma have both been separately shown to predict loss of asthma control and increased exacerbation rates^{6, 19, 20}. This is also true of fractional exhaled nitric oxide (FeNO) levels²¹ and airway hyper-responsiveness (AHR)⁶, the latter being largely driven by airway inflammation²². It is therefore logical that one might wish to control inflammation over and above simply controlling symptoms and lung function – much like controlling asymptomatic hypertension to prevent subsequent cardiovascular sequelae. This is relevant given lung function and lack of symptoms may be deemed normal despite the possibility of an ongoing underlying inflammatory process²³.

50 We performed a post-hoc pooled analysis of data from four previously published
51 randomised controlled trials (RCTs) where inhaled corticosteroid (ICS) dose
52 titration was used in a prospective manner. Outcome measurements included
53 symptoms, lung function, inflammation and AHR. We then analysed the dose-
54 response relationship to ICS for these outcomes to identify where incremental
55 ICS dosing provides the greatest impact, and thus likely to be most informative
56 when titrating a given individual's treatment to achieve optimal or total asthma
57 control.

58 **Methods**

59

60 *Patients*

61

62 Male and female mild-moderate, non-smoking, persistent asthmatics aged 18-65
63 years receiving $\leq 1000\mu\text{g/day}$ ICS, i.e. expressed as a reference dose of large
64 particle beclomethasone dipropionate (BDP) equivalent dose, were recruited to
65 each of four RCTs^{11, 24-26}. For example, large particle HFA-fluticasone 200ug or
66 small particle HFA-beclomethasone 200ug would be equivalent to large particle
67 HFA-BDP 400ug. Their post-run-in baseline measurements are presented in
68 Table 1. Further detailed inclusion and exclusion criteria can be found in each of
69 the reported trials.

70

71 *Study Design*

72

73 We performed a post-hoc analysis using data from 4 RCTs^{11, 24-26}, each
74 comprising a component where the effects of ICS dose ramp increments were
75 examined on a variety of outcomes including: spirometry, FeNO, AHR including
76 both indirect (mannitol) and direct (methacholine, histamine) challenges,
77 asthma symptoms, serum eosinophilic cationic protein (ECP), and blood
78 eosinophil count (Eos). Briefly, one study used large particle hydrofluoroalkane
79 (HFA)-fluticasone propionate (FP) using FeNO as the primary outcome²⁴: with
80 an ICS-free run-in, followed by either 200 μg or 800ug BDP equivalent. A second
81 study titrated small particle HFA-ciclesonide against mannitol AHR versus
82 titration using standard British Thoracic Society (BTS) guidelines¹¹: patients

were selected from both arms of this study where there was an appropriate dose ramp (i.e. BDP equivalent 200-800µg/day). A third study used methacholine AHR as the primary outcome comparing large particle (HFA) and chlorofluorocarbon (CFC) formulations of budesonide²⁵: within the HFA arm there was an ICS free washout followed by 200µg/day or 800ug/day BDP equivalent. The fourth study examined whether propranolol was useful as an ICS sparing agent²⁶, with histamine AHR the primary outcome: the control arm was effectively a dose ramp between small particle HFA-beclomethasone at BDP equivalent doses of 200µg/day or 800ug/day.

Measurements

Extended detail of measurements can be found in each parent study. Briefly, spirometry was measured using a SuperSpiro spirometer (Micro Medical Ltd.) according to American Thoracic Society (ATS) guidelines²⁷. Exhaled tidal nitric oxide was measured according to ATS recommendations²⁸ using a NIOX analyser (NIOX® Nitric Oxide Monitoring System, Aerocrine AB) prior to other pulmonary function measures. Mannitol challenge was performed as previously described²⁹ with a dry powder inhaler (Aridol; Pharmaxis Ltd) using cumulative dose increments up to 635 mg. Histamine for bronchial challenge was dispensed via nebulized solution with doubling concentrations of histamine from 0.3125mg/ml to 40mg/ml. Methacholine challenge was performed using the five-breath dosimeter technique in accordance within ATS recommendations³⁰. Peripheral blood eosinophils were measured using the Sysmex XE2100 Hematology auto-analyser. Serum ECP was measured in duplicate using a

UniCAP system (Phadia) with a coefficient of variation of 3%. For symptoms, 3 studies^{11, 24, 26} included the mini-Asthma Quality of Life Questionnaire (AQLQ, symptom component), where a mean score of 7 indicates no symptoms, and <7 indicates progressively worse symptoms. In the fourth study²⁵, the following rating scales were used: 0, no asthma symptoms; 1, mild symptoms (easily tolerable); 2, moderate symptoms (interferes with normal activities/sleep); and 3, severe (prevents normal activities/ sleep). The total asthma symptom score was a mean of both morning and evening symptom scores.

Statistical Analysis

All data were initially assessed for normality of distribution, with non-Gaussian distributions logarithmically transformed to enable parametric analyses. For the primary analyses, examining for any change within a given outcome measure following an ICS dose ramp, arithmetic means of the difference within each ICS dose ramp were calculated for outcomes with the same parametric measure across all 4 studies. Geometric mean fold differences were calculated for changes in outcomes that were either non-parametric, or with different measurements between the studies in order to standardize any changes (e.g. bronchial challenges). Analyses of variance (ANOVA) with Bonferroni correction were also used to compare the differences between dose ramp responses, and each individual dose mean. Multiple linear regression analyses, using both forward stepwise and non-hierarchical introduction of predictors, were also employed to examine for any confounding effects of biological covariates on the

main outcome measures. Statistical significance for all comparisons was set at $P < 0.05$. Statistical analyses were performed using IBM SPSS version 22.

Ethics

The East of Scotland Research Ethics Service granted ethical approval for all studies (Refs: NFB/FB/192/03¹¹, 09/S0501/52²⁴, 11/ES/0031²⁶, 08/S1402/14²⁵). All patients provided written informed consent. The studies were registered at ClinicalTrials.gov: NCT00667992²⁵, NCT00995657²⁴, NCT01216579¹¹, NCT01544634²⁶.

Results

We included 121 evaluable participants from the parent studies (Table 1). Ciclesonide patients were approximately 10 years older. Patients had generally preserved pulmonary function, overall mean FEV₁ 85.1% predicted, and had mild symptoms. FeNO was higher in the Fluticasone group due to the inclusion criteria of that study. Despite the different bronchial challenges, their figures all indicated a moderate-severe degree of AHR. Patients had been receiving similar ICS doses prior to study inclusion, mean 420µg/day BDP equivalent.

For pulmonary function, there were small but statistically significant changes seen within the 0-200µg dose ramp for both FEV₁ and FEF₂₅₋₇₅ (Table 2, Figure 1), with a 3.3% (95%CI 2.0,4.7) rise in FEV₁ (P<0.0001), and 4.6% (95%CI 2.4,6.9) rise in FEF₂₅₋₇₅ (P<0.0001). However, there was a plateau in response at 200-800µg for both these measures. There were also statistically significant within-group improvements for symptom scores (Figure 1) at all dose ramps, but with less improvement within the 200-800ug dose ramp: along with a significant difference (P=0.01) when comparing responses between 0-800µg vs. 200-800µg, i.e. again indicating a plateau in response.

For inflammation, significant improvements in FeNO were seen across all ICS dose ramps (Table 2, Figure 2a), with clear evidence of dose separation (Table 2). Improvements were more pronounced in the subgroup with baseline values of FeNO≥25ppb (Figure 2b), and significantly different compared to the subgroup with baseline values of FeNO<25ppb. Serum ECP did not improve with

the low dose ramp 0-200 μ g (Table 2, Figure 2c), rather requiring the higher ICS dose to achieve significant within-group improvements between 0-800 μ g ($P=0.002$) and 200-800 μ g ($P=0.0002$); again the dose ramp responses were significantly different between 0-200 μ g and 200-800 μ g ($P<0.05$). Finally there were significant within group improvements for blood Eos across all dose ramps (Table 2, Figure 2d), where Eos also continued to fall significantly as the ICS dose increased: 370cells/ μ L (95%CI 280,450) at 0 μ g, to 250cells/ μ L (95%CI 200,300) at 800 μ g, $P=0.03$.

Significant within group changes were seen across all dose ramps for AHR (Figure 3a). The greatest improvement was in the 0-800 μ g group at 1.35 doubling dilutions (DD) (95%CI 1.06,1.63), $P<0.0001$, with further significant improvement in the 200-800 μ g group amounting to 0.7DD (95%CI 0.43,0.96), $P<0.0001$. Significantly greater improvements were seen when AHR was separated into indirect (mannitol) versus direct challenges (histamine, methacholine), particularly at the lower dose ramp 0-200 μ g, 1.64DD (95%CI 0.94,2.35) indirect vs. 0.65DD (95%CI 0.40,0.89) direct, $P=0.015$ (Figure 3b).

Multiple linear regression analyses were performed on four main outcome measures: change in FEV₁ (% predicted); change in symptom scores; change in FeNO levels; and AHR doubling dilution differences. The covariates used as predictors of these outcomes were: age; gender; and ICS dose ramps (i.e. 0-200 μ g, 0-800 μ g and 200-800 μ g). ICS dose ramps significantly predicted all outcomes in keeping with our previous findings: change in FEV₁% ($p<0.001$); change in symptoms ($p=0.012$); change in FeNO ($p<0.001$); and change in AHR

193 (p=0.003). Age was not a significant predictor of any outcome measure. Gender
194 was a significant predictor of change in symptom scores (p=0.001), suggesting
195 male gender correlated with a greater improvement in symptoms, but gender
196 did not significantly impact on changes in FEV₁%, FeNO or AHR.
197

Discussion

In the present study we have demonstrated that incremental ICS dosing in persistent asthma leads to small improvements in both pulmonary function and symptoms, which then reaches a plateau above low doses. We have also found that the same ICS dose ramps reveal further room for improvement in both inflammatory outcomes and AHR, when using higher ICS doses up to 800µg/day (beclomethasone equivalent).

The BTS guidelines describe the goal of total (or optimal) asthma control as comprising no symptoms day or night, normal lung function and no exacerbations⁵. Unfortunately, it has been shown that patients are not good at recognizing when their asthma is not controlled³. Furthermore, lingering underlying inflammation may be present²³, and until this is treated, symptoms may take many months to become completely normal³¹. We have shown here that patients who have only mild pulmonary function and symptom impairment respond optimally to low doses of ICS for these outcomes. Pointedly, however, the 3.3% rise in FEV₁ for 0-200µg equated to a mean of 98ml (95%CI 57,140), which is less than the putative minimal clinically important difference (MCID) of 230ml in asthma³². Moreover, for symptoms, the geometric mean fold difference of 0.94 (95%CI 0.90,0.98) in the 200-800µg group equates to around a 6% improvement. For the mini-AQLQ, for example, 6% represents a change of around 0.4, where the MCID is 0.5³³. The low dose plateau of symptoms and lung function have been well documented³⁴⁻³⁷; for example, Masoli et al. showed no further improvement in pulmonary function beyond 200µg day of FP³⁷. Indeed

Pauwels et al. in the FACET study³⁸ showed a dose response on exacerbations but not FEV₁ comparing 200µg/day vs. 800µg/day of budesonide. Yet we still largely base our asthma management on these two outcomes, in addition to reliever use, particularly for mild to moderate persistent asthma; thus limiting further potentially appropriate ICS escalation.

We have demonstrated room for further improvement in multiple markers of inflammation (FeNO, serum ECP and blood Eos) and AHR with higher doses of ICS, despite the an apparent plateau in symptoms and lung function. The presence of ongoing airway inflammation in asthma has been shown to predict not only future exacerbations^{21, 39}, but also loss of asthma control²⁰ upon reduction or removal of ICS treatment. Furthermore, this has been demonstrated using a variety of inflammatory outcomes including sputum Eos^{20, 39}, blood Eos¹⁶, FeNO²¹ and AHR^{11, 13}. Moreover, targeted ICS therapy towards inflammatory outcomes has been shown to reduce the rate of exacerbations¹⁰, or reduce the overall steroid dose required for total control¹². We have found that while most improvement in FeNO occurs with low dose ICS, there is still a further dose response to higher ICS doses, particularly in patients who exhibit a high baseline FeNO $\geq$ 25ppb. This dose response has been shown previously, and indeed when ICS is stopped there is a rebound rise in FeNO once again⁴⁰, which alludes to a possible need for persistent over intermittent ICS therapy, or indeed non-adherence to ICS therapy⁴¹. Indeed, in the FeNOtype study²⁴ (one of the parent studies of this cohort), significant improvements in asthma control were seen in the ACQ scores, where patients moved from 'not well controlled' to 'well

controlled' with an ACQ <0.75, upon steroid titration that significantly reduced the patients' FeNO levels.

We saw a similar pattern of response to that of inflammatory outcomes with AHR to a variety of bronchial challenges, both direct and indirect. Indirect challenges are more closely related to the inflammatory pathway as they invoke the inflammatory response in the airway to cause bronchoconstriction²². This is perhaps the reason we found a greater magnitude of response for AHR to indirect than direct challenges, in keeping with previous studies^{42, 43}. Targeting ICS therapy using mannitol challenge AHR has been shown to reduce mild exacerbations over and above standard guideline driven therapy¹¹, in line with that for sputum eosinophils¹⁰. However, AHR can be driven by other mechanisms such as airway smooth muscle hyperplasia, and airway closure itself⁴⁴, and is therefore an area requiring further study with regards to personalised asthma treatment⁴⁵.

There is therefore a growing body of evidence suggesting that we need to include inflammatory measurements routinely to best manage patients with asthma. This is problematic, not least due to the cumbersome nature of inflammatory and challenge measurements that are difficult to perform in a community setting; albeit measuring blood eosinophils might be part of the solution¹⁶. Cowan et al. have suggested using a panel of inflammatory biomarkers to better enable prediction of ICS responsiveness in asthma⁴⁶, but delivery of such a test remains the most difficult hurdle. Even simply using the asthma control questionnaire²⁴ itself seems to be a good predictor of future risk⁸.

We believe the strengths of this study are in the cross-section of patients with mild to moderate disease that we commonly see in clinical practice; who additionally use a variety of ICS moieties. Furthermore we believe it has been helpful to study a wide variety of outcome measures, none of which have taken priority in the study design. There are significant limitations of this study due to its post-hoc nature, with relatively low numbers and our ability to only examine low to moderate, albeit commonly used, doses of small and large particle HFA-ICS. Additionally, we do not have longer-term outcome data such as any effects on exacerbation rates. The findings of this study can, therefore, only generate hypotheses for larger, longer-term prospective randomised controlled trials. We would suggest that they do indeed focus on both phenotyping patients on study inclusion, as well as examining the impact of combining measures of inflammation and AHR in addition to asthma control when titrating ICS, rather than using single outcome measures.

It is likely that when treating any individual patient in real life that the clinician needs to adopt a multifactorial approach when titrating therapy, using all of the available information in terms of exacerbations, symptoms, pulmonary function, reliever use, FENO, blood eosinophils and AHR. This is likely to have the best predictive value for any given individual in terms of tailoring appropriate ICS and adjunctive controller therapy to achieve the best long term control.

In conclusion, we have demonstrated that there may be further room for improvement in markers of inflammation and AHR, despite a seeming plateau in the dose response to ICS for both asthma symptoms and pulmonary function,

with small and large particle ICS HFA-formulations and doses. This points to a potential unmet need of uncontrolled underlying airway inflammation in certain asthmatic patients, which may be a precursor to future loss of asthma control.

We would like to emphasise that further prospective study is however required to prospectively examine this issue, with particular reference to longer-term outcomes including exacerbation rates and overall asthma control and how this relates to inflammatory markers.

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Study Baselines	All n=121	FP (ICS Free) n=21	Bud (ICS free) n=72	HFA-BDP (200µg BDP) n=16	CIC (200µg BDP) n=12
Age (yr)	39.8 (37.2,42.4)	36.8	39.6	37.8	48.7
Sex (M:F)	44:77	6:15	29:43	6:10	3:9
SPT allergens [§]	3 (2,4)	3 (1,4)	3 (2,3.25)	2.5 (1,4)	1.5 (0,3.25)
SPT positivity (% patients)	89	81	94	88	63
FEV ₁ (% pred)	85.1 (82.9,87.3)	88.5	82	90.3	89
FEF ₂₅₋₇₅ (% Pred)	65.7 (61.5,69.8)	53.5	70.4	60.3	-
FEV ₁ /FVC ratio (%)	75.2 (73.7,76.7)	71.2	75.6	74.6	80.5
FeNO (ppb)*	37.3 (32.3,42.6)	72.4	34.7	29.1	25.4 (n=10)
AHR*	-	102mg (57,183) Mann PD ₁₅	0.72 mg/ml (0.58,0.90) Meth PC ₂₀	1.31mg/ml (0.64,2.69) Hist PC ₂₀	59mg (15,233) Mann PD ₁₀
ECP (µg/L)* (n=47)	20.5 (15.9,26.3)	18.6	-	22.4	21.9
Eos (cells/µL) (n=37)	330 (280,390)	370	-	290	-
Symptom Score	-	5.8 (5.4,6.2)	0.96 [¶] (0.79,1.12)	6.2 (5.9,6.5)	6.1 (5.6,6.5)
Screening ICS (BDP, µg/day)	420 (361,479)	440	414	406	436

Table 1. Baselines post run-in at the given beclomethasone dipropionate (BDP) equivalent doses for: large-particle hydrofluoroalkane (HFA)-fluticasone propionate (FP), large-particle HFA-budesonide (Bud), small-particle HFA-beclomethasone (HFA-BDP), and small-particle HFA-ciclesonide (CIC). **SPT = Skin Prick Test to common allergens.** Overall means (95%CI), leftmost column. Arithmetic means (95%CI), unless stated. *Geometric mean (95%CI). [§]Median number of allergens (interquartile range). Symptom scores are the symptom component of the mini-AQLQ, except Bud. [¶]Total Symptom Score: 0-no symptoms; 1-mild; 2-moderate; and 3-severe.

ICS BDP (µg/day)	ICS Free (a)	200ug (b)	800ug (c)	P-value (ANOVA)
Pulmonary Function				
FEV ₁ (% pred)	83.4 (81.0,85.9) n=93	87.6 [§] (85.4,89.9) n=121	87.9 [§] (86.0,89.9) n=121	0.01
FEF ₂₅₋₇₅ (% pred)	66.6 (62.0,71.2) n=93	69.6 (65.7,73.5) n=109	70.0 (66.1,73.8) n=109	0.47
Inflammatory Outcomes				
FeNO (ppb)*	40.4 (34.7,46.9) n=93	26.8 [#] (23.4,30.2) n=115	20.8 ^{#¶} (18.8,23.1) n=115	<0.0001
ECP (µg/L)*	18.7 (12.1,28.8) n=21	20.2 (15.3,26.5) n=47	13.2 (9.9,17.4) n=47	0.08
Eos (cells/µL)	370 (280,450) n=21	300 (240,350) n=38	250 [§] (200,300) n=38	0.04

Table 2. Pulmonary function and inflammatory outcomes. Data presented as arithmetic means (95% confidence intervals) unless otherwise stated. *Geometric mean (95% CI). [§]P<0.05 vs. (a). [¶] P=0.01 vs. (b). [#]P<0.001 vs. (a).

1 Figure Legends

2 **Figure 1.** Dose Responses for FEV₁ and Symptom Scores. (a) mean percentage changes
3 (95%CI error bars) for FEV₁ as %predicted. (b) change in symptom scores as geometric
4 mean fold differences (GMFDs, 95% CI error bars); scores <1 indicate improvement.
5 Asterisk denotes significant within-group change, P<0.05. Remaining P-values compare
6 responses between groups.

7
8 **Figure 2.** Dose-responses for inflammatory outcomes. Within-group changes (GMFDs,
9 95%CI) unless stated; scores <1 represent a reduction. (a) FeNO. (b) FeNO comparing
10 baseline FeNO≥25ppb (squares) vs. FeNO<25ppb (circles). (c) serum ECP. (d) blood
11 eosinophils, arithmetic mean (95%CI). Asterisk denotes significant within-group
12 difference, P<0.05. Remaining P-values indicate significant between group differences; or
13 significant differences between baseline FeNO groups within each ramp (b), P<0.05.

14
15 **Figure 3.** Dose-responses in airway hyper-responsiveness (AHR). Within-group changes
16 expressed as doubling dilution differences (DDD). Scores >0 indicate improvement. (a)
17 combined bronchial challenges: 0-200, n=93; 0-800, n=93; 200-800, n=120. (b) direct (circles)
18 vs. indirect (squares) challenges: direct 0-200, n=72; direct 0-800, n=72; direct 200-800, n=88;
19 indirect 0-200, n=21; indirect 0-800, n=21; indirect 200-800, n=32. Asterisk denotes significant
20 within group change, P<0.05. Remaining P-values indicate significant between group
21 differences (a), or significant differences between challenge groups within each ramp (b),
22 P<0.05.

23

Figure 1

Figure 2

Figure 3