Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

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ON-LINE SUPPLEMENT

Mepolizumab (anti-IL 5) and exacerbations of refractory eosinophilic asthma

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I. METHODS AND MEASUREMENTS

The fractional exhaled nitric oxide was measured online at a flow rate of 50 ml/sec (Niox chemiluminescence analyser, Aerocrine, Sweden) as the mean of two readings, differing by less than 10% as previously described (1). Spirometry was performed using a rolling seal spirometer (Vitalograph, UK) as the best of two blows within 100 mls and repeated twenty minutes after inhalation of 200μg albuterol where specified. Bronchial provocation testing to methacholine was performed using the tidal breathing method as previously described (2). Long acting β_2-agonist and ipratropium bromide were withheld for 24 hours and short acting β_2-agonist for 6 hours before testing. Doubling concentrations of methacholine were inhaled from 0.03 mg/ml to a maximum concentration of 16mg/ml and the PC_{20} calculated by linear interpolation of the log-concentration response plot. As subjects in the study had severe asthma, the test was performed in a limited subset of participants that had no significant contraindications and a FEV_1 >60% predicted. Sputum induction and processing was performed as previously described (3). Differential cell counts were recorded by a blinded individual and expressed as percentage values of a sample containing at least 400 non-squamous cells. Due to the expected anti-eosinophil effects of mepolizumab, sputum and blood leucocyte differential counts obtained during scheduled and unscheduled visits were not disclosed to blinded investigators.

Symptom scores were recorded using both the modified Juniper asthma control questionnaire (4) and a horizontal 100 mm visual analogue scale for each of the symptoms of cough, breathlessness and wheeze (5). Asthma quality of life was measured using the standardised Juniper asthma quality of life questionnaire (6).

All bronchoscopies were performed by blinded senior clinicians, in accordance with published guidelines (7). During bronchoscopy, subjects had a bronchial wash with 20 ml isotonic saline; six endobronchial biopsies were collected and if tolerated, bronchoalveolar lavage was performed using warmed isotonic saline, administered as three sequential 60 ml boluses into the right middle lobe. The bronchial wash and lavage fluid were processed as previously described. Cytospins stained with Romanowski stain were counted by a blinded individual and cell counts were expressed as a percentage of at least 400 inflammatory cells. Biopsy specimens were processed as previously described and embedded in glycol methacrylate (8). Immunostaining was performed for major basic protein (MBP) and measurements were made by a blinded individual of the number of MBP^+ cells/mm^2 in submucosa and thickness of the subepithelial layer, recorded as the mean of fifty measurements over a distance of at least 1mm, as previously described (9).

Helical thin section computed tomography (CT) scan has been used to assess airway remodelling in patients with asthma (10). Subjects were administered a dose of long acting β_2-agonist within 3 hours of the CT being undertaken. The scan was performed at full inspiration and limited from the aortic arch to the carina, to capture the right upper lobe apical segmental bronchus (RB1). All scans were obtained using the Siemens Sensation 16 mutislice scanner at 0.75mm collimation, 120kV, 50mAs, pitch 1.1, scan length 53 mm and scan time of 2.85 s. Images were reconstructed at 0.75mm slice thickness using a 512x512 matrix and a very sharp reconstruction algorithm (B70-f). RB1 bronchus on the CT images from all subjects was identified and the airway wall cross sectional geometry was measured with a semi-automated program (Emphylyx-J V 1.00.01; British Columbia University, Vancouver) using the full width half maximum (FWHM) technique. Wall area (WA), lumen area (LA),

maximum airway diameter (Dmax) and minimum airway diameter (Dmin) were measured. WA and LA were corrected for size dependant error and oblique orientation as described below. The total area (TA) and percentage wall area (%WA) were derived from the LA and WA (TA = LA + WA; %WA = WA/TA x 100). All airway dimensions were corrected for body surface area.

We designed an airway phantom modelling the right upper lobe ASB (RB1) down to the 12^{th} generation airways to assess the accuracy and repeatability of manual and automated measures of cross-sectional airway geometry and to derive ways of predicting and minimising observer error. We derived correction equations by looking at the best parabolic planar 3 dimensional fit of the phantom tube measured wall area/luminal area, the maximum/minimum diameter of the airway luminal ratio (a marker of oblique orientation) and the true wall area/luminal area measured by stereomicroscopy to the nearest micron. For each tube 7 values of maximum/minimum ratio and corresponding geometry (wall area and luminal area) measured using the full width half maximum (FWHM) method were derived based upon reconstructing each phantom tube at 10º increments from 0º (perpendicular to the long axis of the tube) to 60º corresponding to a ratio of largest to smallest diameter of 1.0 to 2.0. The final correction equations were derived using all 63 measurements of the 9 phantom tubes. Correction equations were generated using a custom program (LeoStatistic, Version 14.5, www.leokrut.com). The correction equations derived from multivariate analysis using parabolic approximation were:

True LA= $20-0.014(\text{Measured LA}-20)^2 + 3.7(\text{Dmax/Dmin}-2.1)^2$ **[r^2=0.85]**

True WA= $50-0.0073(\text{Measured WA}-92)^2 + 7.5(\text{Dmax/Dmin}-2.3)^2$ **[r^2=0.80]**

II. STUDY PROTOCOL

Scheduled visits

These are summarised in supplement figure 1. All subjects had a clinical history, physical examination, FE_{NO}, spirometry and symptom scores at an initial baseline visit. Allergen skin prick tests to *Aspergillus fumigatus* and four common UK aeroallergens (cat fur, dog dander, grass pollen and *Dermatophagoides pteronyssinus*) were performed and blood samples collected to measure total plasma IgE. Parasite serology to toxocara, filariasis and schistosomiasis was performed and all women of child bearing age underwent a urine pregnancy test. At the end of this visit, subjects were issued with diary cards and a self-management plan that was based upon symptoms and PEF measurements (11); they were asked to make no changes to their regular asthma medication until study completion. Subjects attended for visit 2 after a 2-week run in period. Methacholine PC_{20} was measured, followed a day later by baseline FE_{NO}, spirometry, AQLQ and methacholine PC20. Subjects were issued with 2 weeks of oral prednisolone at a dose of 0.5 mg/kg/day to a maximum of 40 mg/day prior to randomisation and first treatment at visit 3. The subgroup of participants consenting to bronchoscopy had the pre-treatment procedure performed shortly after visit 2, prior to commencing prednisolone. At visit 3, after completing the 2-week course of prednisolone and prior to receiving the first study treatment subjects had a baseline assessment of symptom scores and CT; FE_{NO}, spirometry were also measured. Treatment was given at twelve monthly visits between visit 3 and 14, at the same time of day. FE_{NO}, spirometry and symptom scores were recorded at each visit; AQLQ was measured at visits 5, 8, 11 and 14; and methacholine PC_{20} was measured the day before visits 8 and 14. The treatment phase finished 2 weeks after visit 14, 50 weeks after treatment was started. At this point subjects participating in the bronchoscopy sub-study had a bronchoscopy and all subjects were issued with a

further 2-week course of oral prednisolone. Post-prednisolone FE_{NO}, symptom scores and spirometry were measured and CT scans performed two weeks later at visit 15.

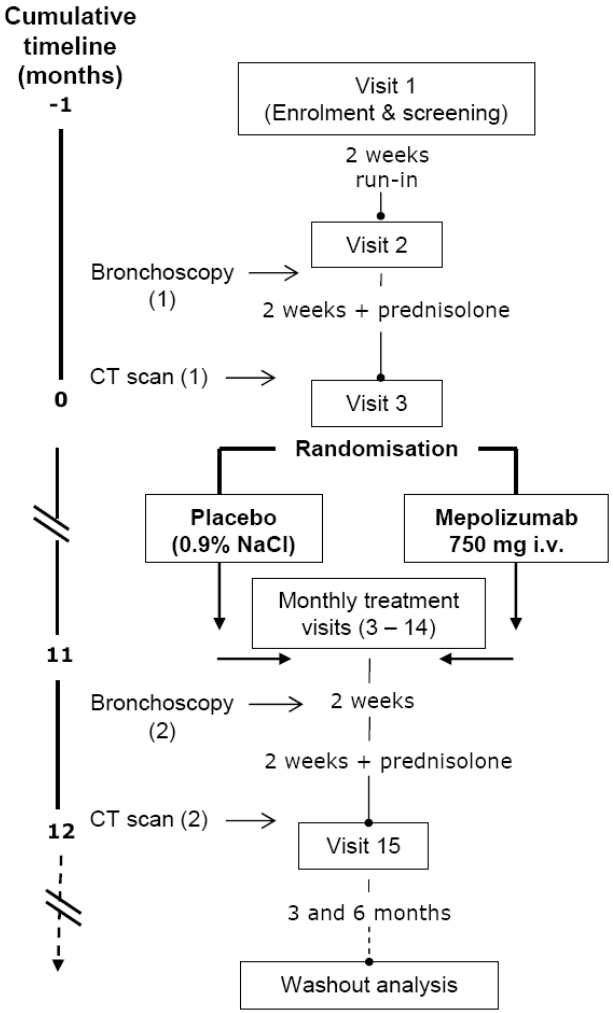
Unscheduled visits

Exacerbation events during the treatment phase of the study were managed in accordance with standard clinical guidelines(12). Subjects initiating treatment themselves in the community did so with guidance from their personalised management plan. In all cases, subjects were instructed to seek medical advice as soon as possible after starting therapy. Management decisions were primarily led by the study physician or the subjects' General Practitioner and included the need for adjunctive therapy such as antibiotics, the duration for which such therapy was continued and the need for hospitalisation. Oral prednisolone therapy was prescribed at a dose of 0.5 mg/kg/day to a maximum of 40 mg/ day. All exacerbation events that required oral prednisolone therapy were recorded by the investigating team.

Subjects reviewed by the investigating team during an exacerbation had a full clinical assessment, spirometry and FE_{NO} performed. Asthma symptoms were recorded as previously described and sputum samples were obtained, when feasible, for cell counts and microbial analysis. However, neither FE_{NO} nor sputum cell counts were available for making treatment decisions. Exacerbation events requiring hospitalisation were managed by the admitting clinical team who were blind to treatment allocation.

III. SUPPLEMENTARY FIGURES AND TABLES

Supplementary figure 1: Overview of study design



	Visit Number	Symptom scores	Asthma quality of life	Bronchoscopy	CT Scan	Spirometry	Exhaled nitric oxide	Sputum induction	Blood leucocyte count	Bronchial provocation test
		Summary of longitudinal measurements								
	1	X				X		X	X	
	2		X	X			X			X
	2-weeks prednisolone									
Treatment Visits	3	X			X	X	X			
	4	X				X	X			
	5	X	X			X	X	X	X	
	6	X				X	X			
	7	X				X	X			
	8	X	X			X	X	X	X	X
	9	X				X	X			
	10	X				X	X			
	11	X	X			X	X	X	X	
	12	X				X	X			
	13	X				X	X			
	14	X	X	X		X	X	X	X	X
	2-weeks prednisolone									
	15	X			X	X	X			

Legend Supplement figure 1

Overview of the study design (left) and a summary of the measurements performed at each study visit (right).

Bronchial provocation testing was performed a day before visits 2, 8 and 14.

Bronchoscopy and CT scanning were performed respectively very soon before and after the scheduled 2-week prednisolone trials.

Supplementary Table 1: Overview and comparison of changes in secondary outcomes after treatment with mepolizumab or placebo

	Mepolizumab		Placebo		Between group difference in change† (95% CI)	Significance ‡
	Baseline	Change from baseline §	Baseline	Change from baseline §		
Fraction exhaled nitric oxide (ppb) *	44.4 ± 0.4	0.85 (0.67 to 1.04)	35.5 ± 0.4	0.99 (0.80 to 1.19)	0.9 (0.6 to 1.1)	0.29
Total sputum neutrophil count (cells per mg selected sputum)	2534 ± 4890	-1291 (-3363 to 779)	1062 ± 1210	370 (-417 to 1157)	-1662 (-4410 to 1085)	0.22
Modified Juniper Asthma Control Score	1.98 ± 1.07	-0.17 (-0.47 to 0.13)	2.38 ± 1.35	-0.21 (-0.52 to 0.11)	0.04 (-0.38 to 0.46)	0.65
Visual analogue symptom score	36.2 ± 22.0	-7.7 (-15.2 to -0.3)	40.6 ± 26.2	-3.2 (-9.0 to 2.7)	-4.6 (-13.9 to 4.7)	0.36
Asthma quality of life score	4.61 ± 1.21	0.55 (0.14 to 0.97)	4.77 ± 0.99	0.19 (-0.06 to 0.44)	0.35 (0.08 to 0.63)	0.02
Post bronchodilator FEV1 (litres)	2.31 ± 0.82	0.06 (-0.09 to 0.21)	2.39 ± 0.85	0.12 (-0.03 to 0.26)	-0.05 (-0.26 to 0.15)	0.61
^Methacholine PC_{20} (mg/ml^{-1}) *	0.6 ± 1.2	0.9 (-1.5 to 2.1)	1.1 ± 1.1	0.4 (-0.6 to 1.5)	2.3 (-0.5 to 0.3)	0.70
Blood eosinophil count ($x10^9$ l^{-1}) *	0.32 ± 0.38	0.15 (0.11 to 0.20)	0.35 ± 0.30	0.9 (0.7 to 1.17)	0.17 (0.11 to 0.24)	<0.001
Sputum eosinophil count (%) *	6.8 ± 0.6	0.14 (0.07 to 0.25)	5.46 ± 0.75	0.51 (0.28 to 0.91)	0.27 (0.12 to 0.63)	0.002
Bronchial wash eosinophil count (%) *	3.1 ± 0.8	0.19 (0.04 to 0.81)	3.1 ± 0.1	3.0 (0.2 to 45.7)	0.06 (0.01 to 0.56)	0.02
Bronchoalveolar lavage eosinophil count (%) *	5.5 ± 0.7	0.1 (0.02 to 0.50)	5.6 ± 0.3	0.8 (0.05 to 12)	0.13 (0.01 to 1.1)	0.06
Bronchial subepithelial eosinophil count (number per unit area) *	47.6 ± 0.4	0.41 (0.03 to 5.3)	10.9 ± 0.5	0.85 (0.04 to 19.1)	0.48 (0.01 to 16.7)	0.68
CT % Wall Area	66.3 ± 4.5	-1.2 (-2.5 to 0.1)	65.0 ± 5.3	-0.4 (-2.1 to 1.4)	-0.8 (-2.9 to 1.3)	0.43

Wall area/ BSA (mm^2m^{-2})	12.1 ± 3.9	-0.6 (-1.3 to 0.1)	11.6 ± 3.9	0.5 (-0.1 to 1.2)	-1.1 (-2.1 to -0.2)	0.02
Luminal area/ BSA (mm^2m^{-2})	6.4 ± 2.8	0.08 (-0.2 to 0.4)	6.5 ± 2.7	0.4 (-0.1 to 0.9)	-0.3 (-0.9 – 0.3)	0.26
Total area/ BSA (mm^2m^{-2})	18.4 ± 6.5	-0.5 (-1.5 to 0.4)	18.0 ± 6.4	0.9 (-0.04 to 1.9)	-1.5 (-2.8 to -0.2)	0.03

Legend Supplementary Table 1:

Mean (SD) pre-treatment values and post-treatment change within and between groups with 95% confidence intervals (CI).

* Geometric mean (log SD) with mean fold change and 95% CI. ^ For methacholine $PC_{20,}$ the change from baseline is expressed as doubling doses.

§ Change was calculated as a difference between the mean or geometric mean of the post treatment values and the baseline values. For parameters expressed as geometric mean, the change is expressed as a fold change.

‡ Significance refers to the between group difference in change.

Asthma quality of life score are obtained using the Standardised Asthma Quality of Life Questionnaire, comprising 32 items on a scale from 1 to 7, grouped into 4 domains. The total score is calculated as the mean score across the 4 domains; an increase in the asthma quality of life score indicates improvement. Symptoms are expressed as a mean of visual analogue scores for cough wheeze and breathlessness, scored on a 100 mm line fixed at both ends by ‘no symptoms’ and ‘worst symptoms ever’. The modified Juniper Asthma Control score is the mean of 5 responses for day and night time symptoms and activity limitation scored on a 0-6 scale; an increase in score indicates worsening asthma symptoms.

18 subjects in the placebo group and 16 subjects in the mepolizumab group had bronchial provocation testing performed. In the mepolizumab group, 9, 8 and 7 subjects had adequate samples for measurement of biopsy samples, bronchoalveolar lavage and bronchial wash at both time-points of the study. The corresponding figures in the placebo group were 5, 3 and 3 subjects.

† The between group difference was calculated as the difference in change from baseline with placebo and mepolizumab.

Abbreviations used: FEV_1 = Forced expiratory volume in 1 second; PC_{20} = Provocative concentration of methacholine required to induce a fall in the FEV_1 of 20% from baseline; CT = Computerised tomography; WA= Wall area; BSA= Body surface area; LA= Luminal area; TA= Total area

Supplementary Table 2: Overview of changes in measures of corticosteroid response with 2-weeks prednisolone before and after 12 months treatment with mepolizumab or placebo.

	Pre treatment		Post treatment		Mean difference in change (95% CI) ^	Significance ‡
	Pre-steroid measurement	Mean change (95% CI) with prednisolone†	Pre-steroid measurement	Mean change (95% CI) with prednisolone†		
Placebo Group						
Modified Juniper Asthma Control Score	2.38 ± 1.25	-0.19 (-0.49 to 0.1)	2.10 ± 1.43	-0.07 (-0.33 to 0.20)	-0.13 (-0.73 to 0.3)	0.40
Post BD FEV1 (litres)	2.39 ± 0.85	0.17 (0.01 to 0.35)	2.56 ± 0.87	0.20 (0.05 to 0.34)	-0.04 (-14.0 to 13.2)	0.84
FE_{NO} (ppb) *	35.6 ± 0.40	0.8 (0.62 to 0.97)	39.8 ± 0.23	0.7 (0.49 to 1.06)	1.3 (0.8 to 2.0)	0.26
Mepolizumab Group						
Modified Juniper Asthma Control Score	1.91 ± 1.09	-0.3 (-0.60 to 0.00)	1.62 ± 1.19	-0.15 (-0.50 to 0.21)	-0.12 (-0.4 to 0.15)	0.37
Post BD FEV1 (litres)	2.3 ± 0.82	0.07 (-0.07 to 0.22)	2.38 ± 0.76	0.10 (-0.03 to 0.23	-0.03 (-21.3 to 20.7)	0.90
FE_{NO} (ppb) *	44.4 ± 0.40	0.6 (0.50 to 0.76)	34.5 ± 0.33	0.7 (0.48 to 0.89)	0.9 (0.5 to 1.6)	0.74

Legend supplementary table 2:

Mean (SD) values for measurements prior to receiving prednisolone. * Geometric mean (log SD) for corresponding FE_{NO}.

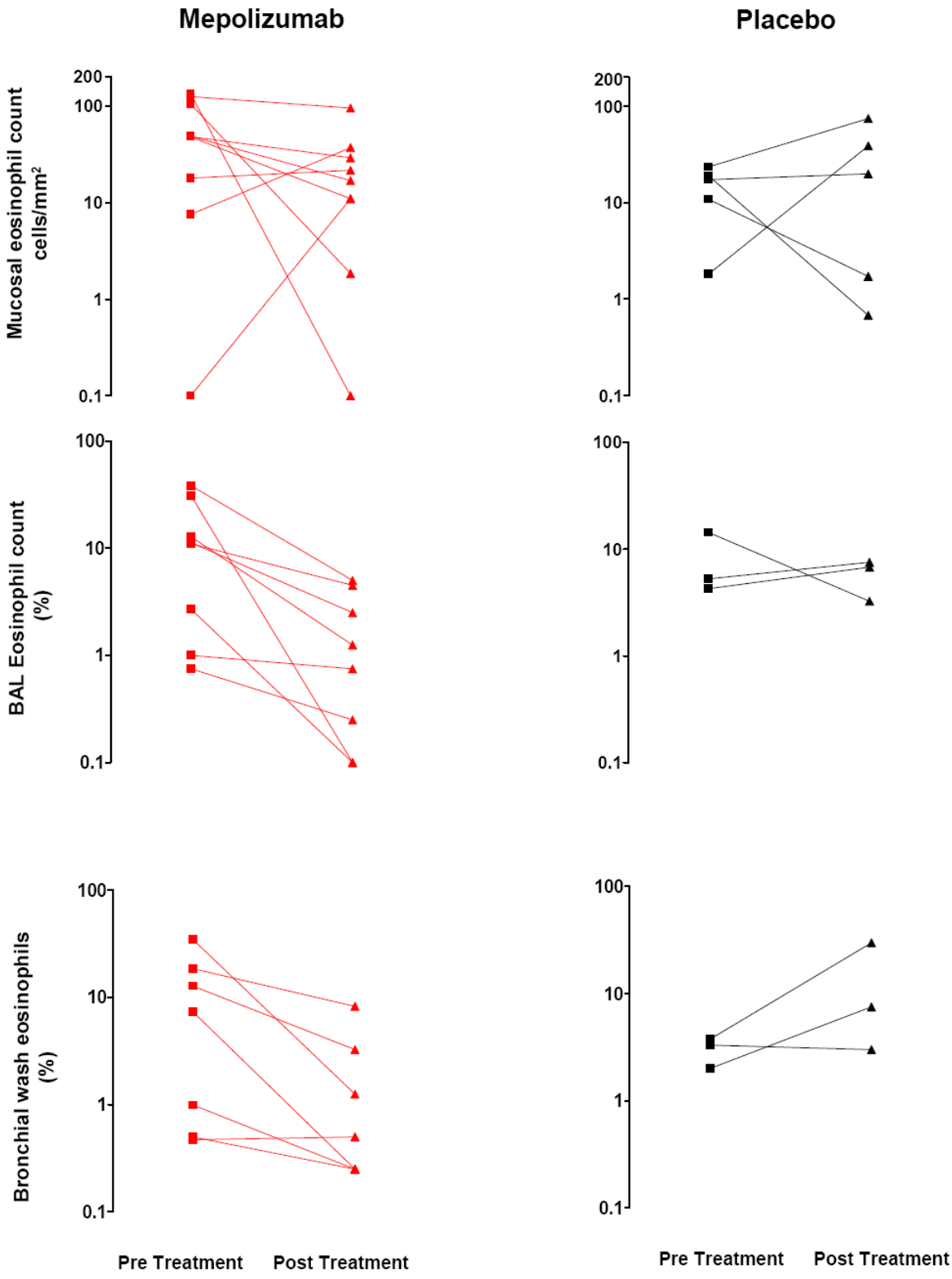
† Change was calculated as a difference between the mean or geometric mean of the post prednisolone values from the baseline values. For $FE_{NO,}$ the change is expressed as a fold change. ^The mean difference in change between post treatment and pre treatment changes with prednisolone in each group. For $FE_{NO,}$ the difference is expressed as a fold change.

‡ Significance refers to the difference in change between the response to prednisolone pre and post treatment.

The geometric mean sputum eosinophil counts before prednisolone pre and post treatment were: 5.8% and 3.2% in the placebo group and 6.5% and 0.6% in the mepolizumab group.

The modified Juniper Asthma Control score is the mean of 5 responses for day and night time symptoms and activity limitation scored on a 0-6 scale; an increase in score indicates worsening asthma symptoms.

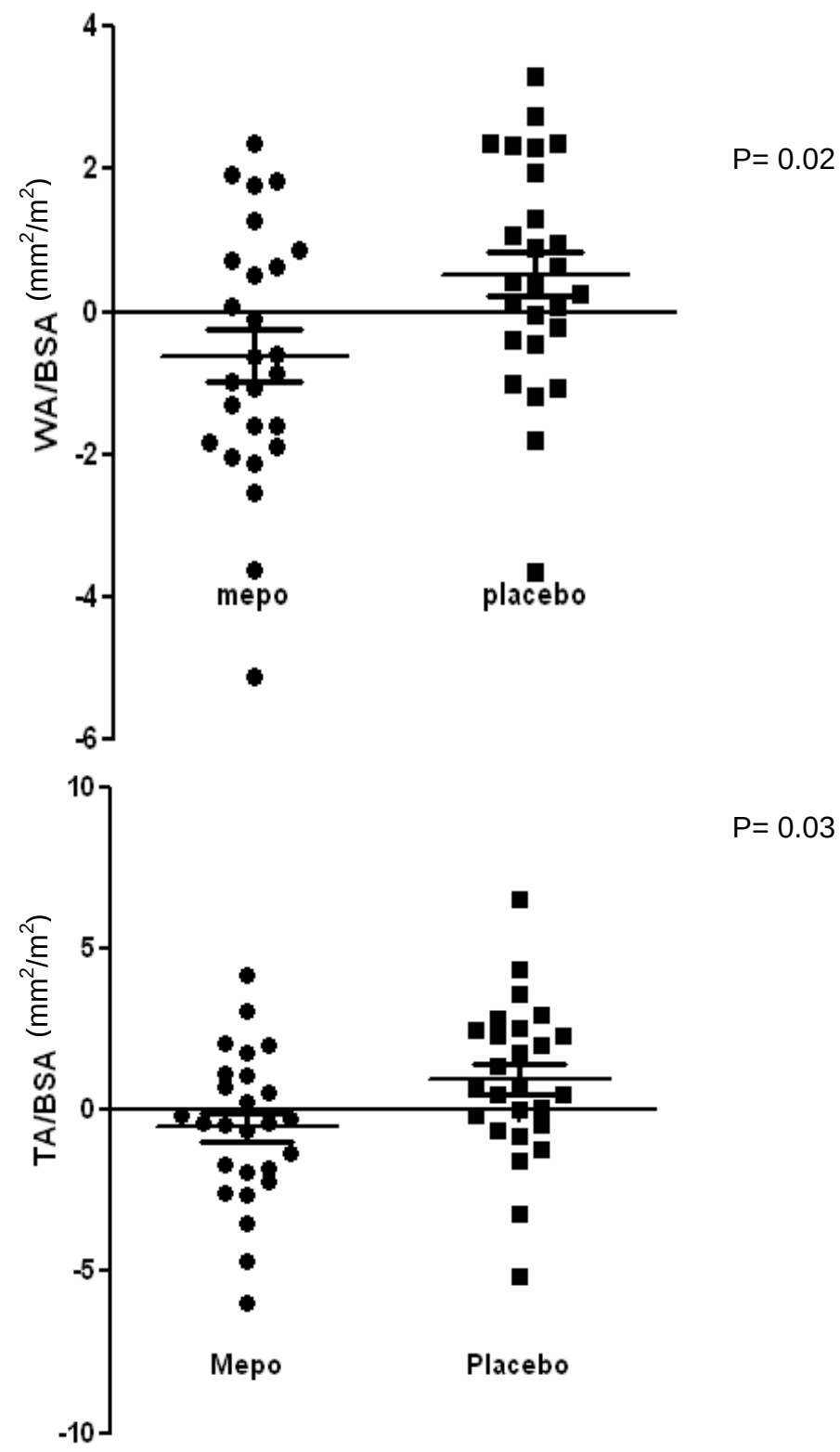
Supplementary figure 2: Eosinophil counts from airway and tissue compartments at bronchoscopy before and after 12 months therapy with mepolizumab or placebo



Legend supplementary figure 2:

Individual bronchial biopsy, bronchoalveolar lavage (BAL) and bronchial wash eosinophil counts before and after mepolizumab and placebo treatment. Bronchoalveolar lavage returns and cell counts were not significantly different within and between groups.

Supplementary figure 3 : Mean change in CT measured wall area (WA) and total area (TA), corrected for body surface area (BSA) after 12 months therapy with mepolizumab or placebo



Legend supplementary figure 3: Horizontal bars represent mean change from baseline and error bars +/- SEM.

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