

Sputum *Streptococcus pneumoniae* is reduced in COPD following treatment with benralizumab

This article was published in the following Dove Press journal:
International Journal of Chronic Obstructive Pulmonary Disease

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Abstract: We hypothesized whether the reduction in eosinophilic airway inflammation in patients with chronic obstructive pulmonary disease (COPD) following treatment with benralizumab, a humanized, afucosylated, monoclonal antibody that binds to interleukin-5 receptor α , increases the airway bacterial load. Analysis of sputum samples of COPD patients participating in a Phase II trial of benralizumab indicated that sputum 16S rDNA load and *Streptococcus pneumoniae* were reduced following treatment with benralizumab. However, in vitro, eosinophils did not affect the killing of the common airway pathogens *S. pneumoniae* or *Haemophilus influenzae*. Thus, benralizumab may have an indirect effect upon airway bacterial load.

Keywords: COPD, benralizumab, IL-5, bacterial load, *S. pneumoniae*, *H. influenzae*

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by irreversible airflow obstruction and airway inflammation. Although typically neutrophilic, COPD is eosinophil-predominant in 10%–40% of cases.^{1–3} Increased airway or blood eosinophil counts are associated with a good response to corticosteroids in stable COPD³ and during exacerbations.⁴ Interleukin-5 (IL-5) binds with high affinity to the IL-5 receptor (R) α (IL-5R α) subunit and plays a pivotal role in the differentiation and maturation of eosinophils in the bone marrow and their survival in tissue.¹ In a 1-year randomized placebo-controlled trial of benralizumab,⁵ a humanized, afucosylated, monoclonal antibody that inhibits IL-5R α activation and promotes antibody-dependent cell-mediated cytotoxicity (leading to near complete eosinophil depletion), improvements in lung function and symptoms and reduction in exacerbations were observed in patients with eosinophilic inflammation. However, in non-eosinophilic COPD patients, exacerbation frequency increased following benralizumab treatment vs placebo. Likewise, in a 6-month trial, the IL-5 neutralizing monoclonal antibody mepolizumab⁶ reduced exacerbations vs placebo in COPD patients with an increased blood eosinophil count but resulted in a greater exacerbation frequency in those with a low blood eosinophil count. This finding contrasts with that for asthma for which absence of eosinophilic inflammation is associated with neither benefit nor harm to anti-IL-5(R).¹ Interestingly, the airway microbiome is distinct between COPD patients with vs those without eosinophilic inflammation.⁷ Corticosteroid therapy alters the airway microbiome⁷ and consequently might hinder recovery during exacerbations in patients without eosinophilic inflammation.⁴ Whether this exacerbation relationship to low eosinophil count is genuine and these effects are partly because of attenuation of

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eosinophilic inflammation remain unknown. We, therefore, hypothesized that reduction in eosinophilic airway inflammation following benralizumab treatment increases airway bacterial load.

Methods

Sputum samples were collected from COPD patients participating in the Phase II trial⁵ of benralizumab at 28 days before baseline, at baseline, and 57 and 255 days after receiving benralizumab or placebo. Written informed consent was provided by all patients. The study was conducted in accordance with the Declaration of Helsinki and was approved by the national and local ethics committees (Table S1). Supernatants were stored at -80°C . For those patients who provided adequate sputum supernatant ($>100\ \mu\text{L}$) and ≥ 1 sample before and after therapy, DNA was extracted using QIAamp DNA mini kit (QIAGEN, Hilden, Germany) as per the manufacturer's protocol. Hydrolysis-based TaqMan assays were used to quantify 16S rDNA gene load (Integrated DNA Technologies [IDT], Coralville, Iowa) and *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae* by targeting the *FucP*, *CopB* (Applied Biosystems Life Technologies), and *pneumolysin* (IDT) genes, respectively. Quantification was determined relative to prepared standard curves for each bacterial strain by using Stratagene Mx3000P (Stratagene; La Jolla, CA) (Table S2).

Ex-vivo peripheral blood eosinophils and neutrophils of $>95\%$ purity from healthy individuals were isolated as previously described.⁸ In vitro, *Escherichia coli* (strain PA360), *H. influenzae* (NCTC11872), and *S. pneumoniae* (D39), grown to late-log phase, were incubated alone or with blood eosinophils or neutrophils in triplicate for 1 h at 37°C in the presence of 0.1% (*E. coli*, *H. influenzae*) or 10% (*S. pneumoniae*) non-heat inactivated human serum. We chose serum concentrations that were sublethal for nontypeable *H. influenzae* and *E. coli* based on titration experiments. Colony forming units were enumerated for each condition to determine the bacterial killing effect of each granulocyte.

Statistical analysis was performed using R 3.4.1 (The R Foundation for Statistical Computing) and PRISM (GraphPad; La Jolla, CA). Change in bacterial load over time within the treatment or placebo group was performed by fitting a generalized linear mixed model for each of 16S rDNA, *H. influenzae*, and *S. pneumoniae*. The dependent variables were time, treatment group, and an interaction term, time*treatment group. A random intercept and

a random effect for time were included. Correlations were undertaken between change in bacterial load, forced expiratory volume in 1 s, and symptoms and health status. Comparisons were made between conditions for bacterial killing experiments by 2-way analysis of variance.

Results

Sputum supernatant samples were assessed from 14 benralizumab-treated and 15 placebo-treated patients. Clinical characteristics were similar between the groups (Table S3). The 16S rDNA load decreased following benralizumab treatment but remained unchanged with placebo (Figure 1A and B). The quantity of *S. pneumoniae* in the benralizumab and placebo groups decreased significantly, with the reduction numerically and statistically greater in the benralizumab group (Figure 1C and D). The reduction in 16S rDNA load was associated with a reduction in the quantity of *S. pneumoniae* in the benralizumab group but not in the placebo group (Figure 1C and D). However, there were no significant changes in the quantity of *H. influenzae* (Figure 1E and F) or *M. catarrhalis* (data not shown) in the 2 treatment groups. The reduction in total bacterial load, or quantity of *S. pneumoniae* in the sputum supernatants in benralizumab-treated patients, was not associated with either baseline blood eosinophil count or change in lung function, symptoms or health status following treatment with benralizumab. In contrast with ex-vivo neutrophils, ex-vivo blood eosinophils did not kill *H. influenzae* or *S. pneumoniae* in vitro (Figure 2A–D).

Discussion

Contrary to our hypothesis, we found that 16S rDNA load and the quantity of the common pathogen *S. pneumoniae* decreased following benralizumab treatment. However, this was not associated with clinical outcomes. There was also a small decrease in the quantity of *S. pneumoniae* in the placebo group. In vitro, blood eosinophils did not affect bacterial killing of *S. pneumoniae* and *H. influenzae*, while small effects were observed on *E. coli*, similar to previously published reports.^{9,10} Therefore, the effects observed in vivo are more likely an indirect effect of benralizumab attenuating eosinophilic inflammation. Macrophage efferocytosis of eosinophils⁸ might further impair macrophage phagocytosis of bacteria; therefore, their reduction may improve bacterial clearance. Similarly,

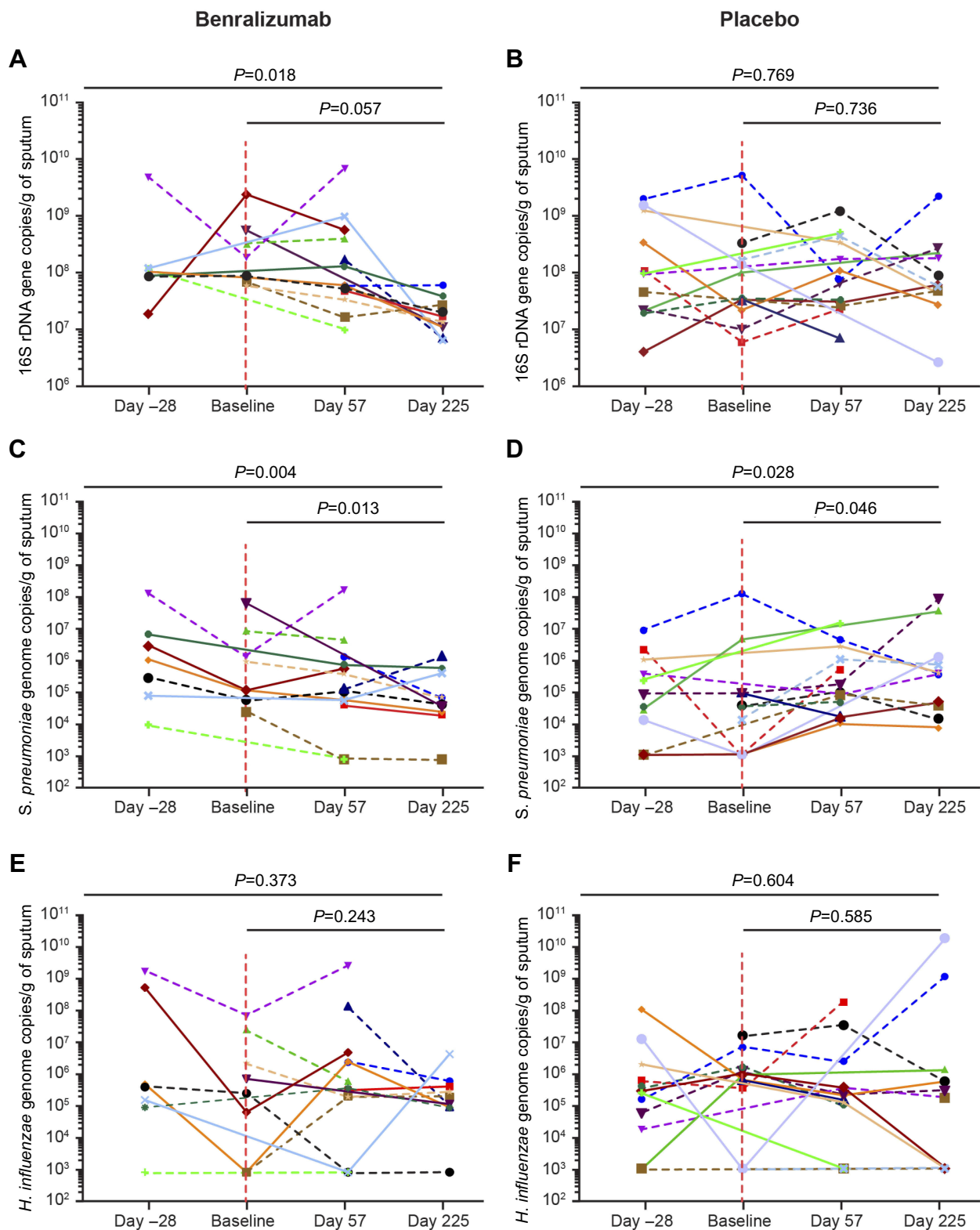


Figure 1 Sputum bacterial load in response to benralizumab vs placebo. Plots for (A and B) total bacterial load, (C and D) *Streptococcus pneumoniae*, and (E and F) *Haemophilus influenzae* for individual patients from Day -28 to Day 225 in response to benralizumab vs placebo. Vertical dotted lines delineate first treatment dose. Horizontal dotted lines represent patients with a baseline sputum eosinophil count $\geq 3\%$. P-values are provided for the mixed models, including all time points, but excluding Day -28.

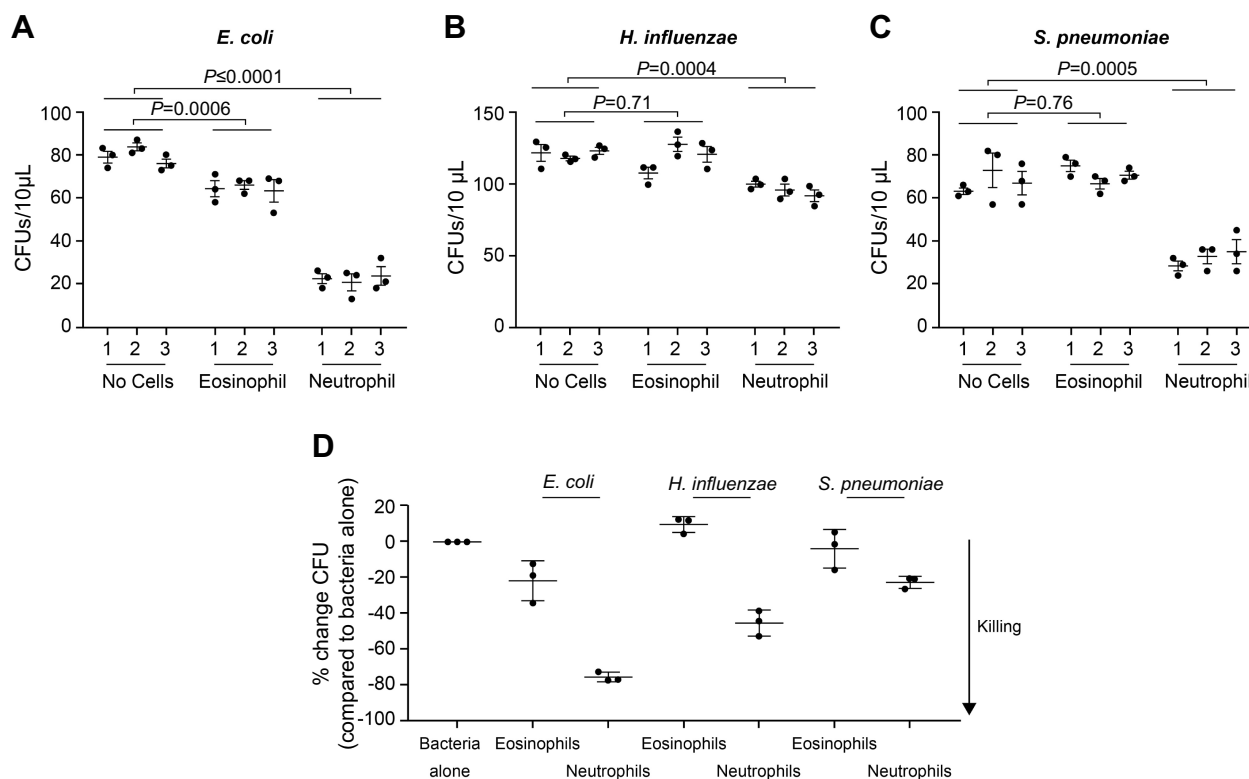


Figure 2 Ex-vivo blood eosinophils do not kill *Haemophilus influenzae* or *Streptococcus pneumoniae* in vitro. *Escherichia coli* (A), *H. influenzae* (B), and *S. pneumoniae* (C) were incubated alone or with purified blood eosinophils or neutrophils, as indicated in triplicate (x-axis), for 1 h at 37 °C, with shaking at 200 rpm in U-bottom plates. Bacteria and cells were incubated in a ratio of ~1:100 in the presence of either 0.1% (A and B) or 10% (C) non-heat inactivated AB-human serum supplemented with RPMI. By removing 3 × 10 µL aliquots from each well and incubating overnight on agar, colony forming units were determined in each 10 µL suspension. Data in A, B, and C represent data from one individual. Data in D represents cumulative data from three separate healthy donors showing % change in CFU/10 µL for each bacterium tested in the presence of eosinophils or neutrophils relative to bacteria alone. A, B, and C bars indicate mean (SEM), with statistics calculated using a 2-way ANOVA (Dunnett's multiple comparison test).

Abbreviations: ANOVA, analysis of variance; CFU, colony forming unit; rpm, revolutions per minute; RPMI, Roswell Park Memorial Institute medium; SEM, standard error of the mean.

reduction in eosinophilic inflammation is likely to result in a greater percentage of neutrophils that might consequently enhance bacterial clearance. Intriguingly, although these possible mechanisms might explain the reduction in bacterial load following benralizumab treatment, they do not provide a rationale for the apparent increased exacerbation risk in non-eosinophilic COPD patients, as observed in the Phase IIa study, following anti-IL-5Rα treatment. Further investigation is needed to assess whether the clinical findings are real and what the underlying cause may be.

The retrospective study design limited the number of available samples, restricted analysis to sputum supernatants rather than whole sputum plugs, and limited us to targeted quantitative polymerase chain reactions rather than broader sequencing approaches. Future prospective studies, including whole sputum and microbiome sequencing, are required to further determine the effects of anti-IL-5/anti-IL-5Rα therapies on airway ecology.

Conclusions

Sputum 16S rDNA and *S. pneumoniae* bacterial load are reduced in COPD patients following benralizumab treatment. However, how biologics affect the airway microbiome in obstructive lung diseases warrants further investigation.

Abbreviation list

COPD, chronic obstructive pulmonary disease; IDT, Integrated DNA Technologies; IL-5, interleukin-5; IL-5Rα, IL-5 receptor alpha; R, receptor.

Acknowledgments

The authors thank Professor Peter Andrew and Dr Vitor Fernandes, of the University of Leicester for their assistance with the in vitro bacterial experiments. Editorial support was provided by Cactus Communications and by Michael A Nissen, ELS, of AstraZeneca. This support was funded by AstraZeneca. This work was funded in part by Airway Disease

PRedicting Outcomes through Patient Specific Computational Modelling (AirPROM) project (funded through the European Union's Seventh Framework Programme [FP7] grant), the National Institute for Health Research (NIHR) Leicester Respiratory Biomedical Centre, and MedImmune Ltd. This paper presents independent research funded by the NIHR. The views expressed are those of the authors and not necessarily those of the National Health Service, the NIHR, or the Department of Health.

Author contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

RVDM is an employee of MedImmune LLC, the manufacturer of benralizumab. UM and PN are employees of AstraZeneca, the manufacturer of benralizumab. UM reports holding shares in AstraZeneca Pharmaceuticals. CEB report grants and personal fees from AstraZeneca/MedImmune LLC, during the conduct of the study. The authors report no other conflicts in this work.

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Supplementary materials

Table S1 List of institutional review boards/independent ethics committees

Site number	Investigator name	Name and address of IRB/IEC
I178101	Robert Cowie	Office of Medical Bioethics, Heritage Medical Research Clinic, 3300 Hospital Dr. NW, Calgary, Canada
I211701	Francois Maltais	L'Institut de cardiologie et de pneumologie deQuebec, 2725 Chemin Ste-Foy, Quebec, Canada
I224301	Kieran Killian	Hamilton Health Sciences, 293 Wellington St. N, Hamilton, Canada
I286101	Andre Frechette	Centre de Recherche Inc., 205 Montmagny St, Quebec, Canada
I254401	Darcy Marciniuk	Royal University Hospital, 1103 Hospital Drive, Saskatoon, Canada
I207401	Guy Chouinard	372 Hollandview Trail, Suite 300, Aurora, Ontario, Canada
I214701	Ingrid Titlestad	Den Videnskabsetiske Komite for Region, Syddanmark, Regionshuset Damhaven 12, Vejle, Denmark
I214801	Vibeke Backer	Den Videnskabsetiske Komite for Region, Syddanmark, Regionshuset Damhaven 12, Vejle, Denmark
I214901	Ronald Dahl	Den Videnskabsetiske Komite for Region, Syddanmark, Regionshuset Damhaven 12, Vejle, Denmark
I227001	Niels Seersholm	Den Videnskabsetiske Komite for Region, Syddanmark, Regionshuset Damhaven 12, Vejle, Denmark
I286301	Jesper Sonne	Den Videnskabsetiske Komite for Region, Syddanmark, Regionshuset Damhaven 12, Vejle, Denmark
I086301	Roland Buhl	Antrag (AMG/multi) - 837.364.10(7373), Landesärztekammer Rheinland-Pfalz EC, Deutschhausplatz 3, Mainz, Germany
I285801	Oliver Kornmann	Ethikkommission (EC) der Landesärztekammer Hessen, Im Vogelsgesang 3, Frankfurt, Germany
I077101	Christopher Brightling	NRES Committee East Midlands – Leicester, The Old Chapel Royal Standard Place, Nottingham, UK
I221301	William MacNee	NRES Committee East Midlands – Leicester, The Old Chapel Royal Standard Place, Nottingham, UK
I221201	David Lomas	NRES Committee East Midlands – Leicester, The Old Chapel Royal Standard Place, Nottingham, UK
I176001	David Singh	NRES Committee East Midlands – Leicester, The Old Chapel Royal Standard Place, Nottingham, UK
I129701	Piotr Kuna	Komisja Bioetyki Uniwersytetu Medycznego, w Łodzi, Al. Kościuszki 4, Łódź, Poland

(Continued)

Table S1 (Continued).

Site number	Investigator name	Name and address of IRB/IEC
1083301	Ewa Jassem	Komisja Bioetyki Uniwersytetu Medycznego, w Łodzi, Al. Kościuszki 4, Łódź, Poland
1285701	Grazyna Pulka	Komisja Bioetyki Uniwersytetu Medycznego, w Łodzi, Al. Kościuszki 4, Łódź, Poland
1078601	Jan Kus	Komisja Bioetyki Uniwersytetu Medycznego, w Łodzi, Al. Kościuszki 4, Łódź, Poland
1229201	Pawel Gorski	Komisja Bioetyki Uniwersytetu Medycznego, w Łodzi, Al. Kościuszki 4, Łódź, Poland
1215001	Pere Casan Clara	Comité Ético de Investigación Clínica de Asturias, Comité Ético de Investigación, Clínica Celestino Villamil s/n Edificio Centro, de Rehabilitación- 5ª planta, Oviedo, Spain
1215201	Ferran Barbe Illa	EC Hospital de Lleida Arnau de Vilanova, Comité Ético de Investigación Clínica Avda., Alcalde Rovira Roure 80 Att. Montse, Solamilla, Lérida, Spain
1284901	David Ramos Barbon	Hospital de la Santa Creu e Sant Pau CEIC, Comité Ético de Investigación Clínica San Antoni Mª Claret 167-Pabellón 19 Servicio de Farmacología Clínica Att. Marcela Domínguez Barcelona, Spain
1291501	Jose Luis Velasco Garrido	Hospital Universitario Virgen de la Victoria Comité Ético de Investigación Clínica Campus Universitario Teatinos s/n Unidad de Calidad Málaga, Spain Comité Autonómico de Ensayos Clínicos de Andalucía CAEC Comité Ético de Investigación Clínica Avda. de la Innovación s/n, Edificio Arena 1 Secretaría del CEIC-Consejería de Salud/Sevilla, Spain
1288401	Krishna Pudi	Schulman Associates Institutional Review Board, 4290 Glendale - Milford Road, Cincinnati, Ohio, USA
1288501	James Stocks	Uni of TX Health Sciences Center, Center for Clinical Research 11937 US, Highway 271, Tyler, Texas, USA
1288601	David Fuentes	Schulman Associates Institutional Review Board, 4290 Glendale - Milford Road, Cincinnati, Ohio, USA
1270301	Reynold Panettieri	Schulman Associates Institutional Review Board, 4290 Glendale - Milford Road, Cincinnati, Ohio, USA
1196401	Ritsu Kuno	Schulman Associates Institutional Review Board, 4290 Glendale - Milford Road, Cincinnati, Ohio, USA

(Continued)

Table S1 (Continued).

Site number	Investigator name	Name and address of IRB/IEC
I29000I	Gerard Criner	Schulman Associates Institutional Review Board, 4290 Glendale - Milford Road Cincinnati, Ohio, USA
I29700I	Wesley Bray	Schulman Associates Institutional Review Board, 4290 Glendale - Milford Road, Cincinnati, Ohio, USA
I13620I	Nicholas Nayak	Schulman Associates Institutional Review Board, 4290 Glendale - Milford Road, Cincinnati, Ohio, USA
I29720I	Chaim Bernstein	Schulman Associates Institutional Review Board, 4290 Glendale - Milford Road, Cincinnati, Ohio, USA
I30230I	Michelle Zeidler	VA Medical Center, 16111 Plummer Street, Sepulveda, California, USA
I19690I	Clinton Corder	Schulman Associates Institutional Review Board, 4290 Glendale - Milford Road, Cincinnati, Ohio, USA

Abbreviations: IEC, independent ethics committee; IRB, institutional review board; UK, United Kingdom; USA, United States of America.

Table S2 List of primers used in the study

Primer/probe	Target organism (gene)	Sequence	Reference	Source
S.pneumF	<i>Streptococcus pneumoniae</i> (Pneumolysin)	5'-AGC GAT AGC TTT CTC CAA GTG G-3'	Greiner et al, ¹ 2001	IDT
S.pneumR	<i>Streptococcus pneumoniae</i> (Pneumolysin)	5'-CTT AGC CAA CAA ATC GTT TAC CG-3'	Greiner et al, ¹ 2001	IDT
S.pneum probe	<i>Streptococcus pneumoniae</i> (Pneumolysin)	5'-5CY5-ACC CCA GCA ATT CAA GTG TTC GCG-3BHQ2-3'	Greiner et al, ¹ 2001	IDT
M.catF	<i>Moraxella catarrhalis</i> (outer membrane protein CopB)	5'-GTG AGT GCC GCT TTA CAA CC-3'	Greiner et al, ² 2003	IDT
M.catR	<i>Moraxella catarrhalis</i> (outer membrane protein CopB)	5'-TGT ATC GCC TGC CAA GAC AA-3'	Greiner et al, ² 2003	IDT
M.cat probe	<i>Moraxella catarrhalis</i> (outer membrane protein CopB)	5'-6FAM-TGC TTT TGC AGC TGT TAG CCA GCC TAA-MGBNFQ-3'	Greiner et al, ² 2003	Applied Biosystems Life Technologies
H.inflF	<i>Haemophilus influenzae</i> (fucP)	5'-GCC GCT TCT GAG GCT GG-3'	Price et al, ³ 2015	Eurofins Genomics
H.inflR	<i>Haemophilus influenzae</i> (fucP)	5'-AAC GAC ATT ACC AAT CCG ATG G-3'	Price et al, ³ 2015	Eurofins Genomics
H.infl probe	<i>Haemophilus influenzae</i> (fucP)	5'-6FAM-TCC ATT ACT GTT TGA AAT AC-MGBNFQ-3'	Price et al, ³ 2015	Applied Biosystems Life Technologies
M. Nad16sF	Total bacteria (16S rDNA)	5'-ACT CCT ACG GGN GGC NGC A-3'	Nadkarni et al, ⁴ 2002	IDT
M. Nas16sR	Total bacteria (16S rDNA)	5'-GGA CTA CCA GGG TAT CTA ATC CTG TT-3'	Nadkarni et al, ⁴ 2002	IDT
M. Nad16s probe	Total bacteria (16S rDNA)	5'-56-FAM- CGT ATT ACC GCG GCT GCT GGC AC-36-TAMSp-3'	Nadkarni et al, ⁴ 2002	IDT

Abbreviations: H.infl, *Haemophilus influenzae*; IDT, Integrated DNA Technologies; M.cat, *Moraxella catarrhalis*; rDNA, recombinant deoxyribonucleic acid; S.pneum, *Streptococcus pneumoniae*.

Table S3 Clinical characteristics of COPD patients

Parameters	Benralizumab N=14	Placebo N=15	P-value
Sex (male), n	10	12	0.682
Age, years	64 (10)	65 (6)	0.782
Smoking history, pack-years	46 (23)	47 (22)	0.899
BMI, kg/m ²	28 (5)	28 (5)	0.978
Exacerbations in last year	0.64 (0.73)	0.44 (0.47)	0.395
6MWD, metres	466 (135)	336 (122)	0.011
BODE index	2 (2)	3 (3)	0.634
SGRQ total	43 (20)	44 (18)	0.940
SGRQ symptoms	63 (22)	57 (24)	0.482
SGRQ activity	54 (28)	50 (24)	0.674
SGRQ impacts	30 (21)	35 (18)	0.443
VAS dyspnea	33 (30)	39 (29)	0.583
VAS cough	34 (27)	37 (26)	0.829
VAS sputum	35 (20)	38 (28)	0.765
VAS purulence	27 (31)	13 (18)	0.1520
Pre-bronchodilator FEV ₁ , L	1.26 (0.52)	1.50 (0.55)	0.249
Post bronchodilator FEV ₁ , L	1.37 (0.56)	1.59 (0.53)	0.299
FEV ₁ % predicted	46 (15)	50 (17)	0.147
FEV ₁ /FVC ratio	47 (9)	50 (10)	0.403
RV, L	3.93 (1.32)	3.51 (1.30)	0.407
TLC, L	7.30 (2.03)	6.50 (2.21)	0.331
DLCO, %	80.2 (26.2)	88.2 (24.1)	0.409
Blood eosinophils/ μ L	230 (190)	210 (140)	0.789
Sputum eosinophil, % ^a	5.1 (2.4, 5.9)	4.1 (0.9, 19.3)	0.991

Notes: Data are presented as mean (SD) unless otherwise stated. ^aMedian (interquartile range).

Abbreviations: BMI, body mass index; BODE, body mass index airflow obstruction, dyspnoea, and exercise; COPD, chronic obstructive pulmonary disease; DLCO, diffusing capacity of the lung for carbon monoxide; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; RV, residual volume; SD, standard deviation; SGRQ, St. George's respiratory questionnaire; TLC, total lung capacity; VAS, visual analog scale; 6MWD, 6-min walk distance.

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