

Online Supplementary Material

**Long-term effects of inhaled corticosteroids on sputum bacterial and viral loads in COPD**

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## Methods

### Study design

Consecutive COPD patients, according to GOLD definition [1], were screened for eligibility among the cohort of patients visiting the outpatient clinic of the Research Centre on Asthma and COPD, University of Ferrara, Italy. Sixty COPD patients, with stable moderate airflow limitation ( $50 \leq$  post-bronchodilator FEV1  $< 80\%$  predicted) were recruited to participate in this prospective, randomized, open-label, blinded endpoint (PROBE) study [2, 3]. This is an exploratory proof of concept study to specifically investigate the effect of long-term treatment with an inhaled corticosteroid (fluticasone propionate - FP) added to a long acting beta-2 agonists (LABA) on sputum bacterial load in stable COPD patients. Steroid naïve COPD patients treated with the long acting  $\beta$ 2 agonists (LABA) salmeterol (SALM) for at least 6 months and free from an exacerbation from at least 3 months were enrolled. No concomitant long acting antimuscarin (LAMA) treatment was allowed. The presence of the following conditions was grounds for exclusion of the recruited COPD patients: asthma, documented concomitant lung diseases (e.g., bronchiectasis or lung cancer), and acute infections of the respiratory tract during the previous 3 months. Medical records were used to capture COPD exacerbations that occurred in the year previous the study and identified as acute episodes requiring treatment with antibiotic agents, systemic corticosteroids, hospitalization, or a combination of these. Only patients with sputum samples available at baseline entered the study. Patients were randomized to receive for 12 months one inhalation twice daily of either SALM [50  $\mu$ g (SALM), n=30] or the combination of a long acting  $\beta$ 2 agonist plus inhaled corticosteroid [salmeterol 50  $\mu$ g plus fluticasone propionate 500  $\mu$ g (SALM/FP), n=30]. Blood cell counts were obtained as routinary test prior to

47 randomization. After baseline evaluation and randomization (visit 0), patients were seen  
48 regularly every 3 months in an outpatient setting: visit 1 occurred in month 3, visit 2 occurred in  
49 month 6, visit 3 occurred in month 9, and the final visit occurred in month 12. Patients were  
50 instructed to contact the center for an unscheduled visit in case of worsening symptoms.  
51 The presence of an exacerbation have been verified and confirmed by a physician of the Centre.  
52 Exacerbation episodes (definition provided below) were treated with antibiotic and systemic  
53 corticosteroids [1]. The following study visit was postponed 3-month after the unscheduled  
54 exacerbations visit. If pneumonia was clinically suspected at any study-time or unscheduled  
55 visit, a chest X-ray was performed.  
56 At each visit, the following procedures were performed: clinical evaluation; lung function  
57 testing; induced sputum collection for inflammatory cell counts, for quantitative and qualitative  
58 bacterial and for respiratory viruses and atypical bacteria detection. The sputum induction  
59 procedure was repeated 1-week after each scheduled visit if the amount and/or quality of the  
60 sputum were not adequate.  
61 The primary outcome was the assessment of bacterial load in the sputum of COPD patients after  
62 12 months of treatment with SALM/FP 50/500 µg twice daily compared to that in COPD  
63 patients treated with SALM µg 50 twice daily. Secondary outcomes included assessment of viral  
64 detection; correlation between sputum viral and/or bacterial load and clinical outcomes  
65 (including lung function, quality of life and exacerbation rate); correlation between changes in  
66 sputum viral and/or bacterial load and sputum/systemic inflammatory cell profiles.  
67 The study conformed to the Declaration of Helsinki, the work was approved by the institutional  
68 ethics committee, and informed written consent was obtained from each subject. This study is  
69 registered with ClinicalTrials.gov, number NCT01213693.

## **Sputum collection and analysis**

Induced sputum was collected and analyzed according to published recommendations [4]. The inflammatory cell counts refer to total non-squamous cells. Sputum samples were considered inadequate, if squamous cells > 20% of total cell count and if cell viability <60% [4]. The sputum sample was split in three aliquots: two aliquots were freshly processed for inflammatory cell count and bacteriology assessments, and the remaining aliquot was immediately stored at – 80°C for use in real-time polymerase chain reaction (PCR) microbial analyses.

## **Quantitative bacteriology**

Sputum samples were processed as previously described [5]. Serial dilutions (up to  $10^{-12}$ ) of sputum samples were made and cultured on the appropriate media. Following incubation, the bacterial colonies were enumerated and subcultured for identification by standard microbiological methods. Colony-forming units (CFU)/mL were calculated. The microbiologic assays (sputum cell cultures) were performed and blindly assessed at the central Laboratory of Clinical Microbiology Department of the Arcispedale S. Anna, Ferrara, Italy. The Laboratory Unit follows a rigorous Quality Assurance (QA) programme for microbial detection and quantification provided by the United Kingdom National External Quality Assessment Service (UK NEQAS - <http://www.ukneqas.org.uk>) and reproducibility assessed for this study. The sputum cell cultures were performed in duplicate and considered acceptable for the analysis if not dissimilar of more than one-fold dilution. If not acceptable, the sampling was repeated 1-week after each scheduled visit. Total bacterial load was expressed as Log<sub>10</sub> CFU/ml of potentially pathogenic and nonpathogenetic total bacterial growth

## **Molecular techniques for sputum microbial and virus profiling**

As internal control to conventional microbial assay, a real time qPCR for selected bacterial pathogens (i.e. *Haemophilus influenzae* and *Streptococcus pneumoniae*) was used as previously described [6].

The commercially available multiplex PCR assay RespiFinder RG® (Qiagen) was used according to manufacture instructions for the simultaneous detection and differentiation of 21 respiratory pathogens, including both viruses and atypical bacteria [7, 8]. The detailed list of the pathogens detectable by RespiFinder is listed in the supplementary table S1.

Commercially available assays that target bacterial 16S rRNA genes and fungal ribosomal RNA genes were used for sputum microbial identification and profiling (Microbial DNA qPCR Arrays BAID-1404ZRR-24 - Respiratory Infections. Qiagen - Venlo, Netherlands). The array contains assays for 41 bacterial or fungal pathogens including *Acinetobacter spp.*, *Aspergillus spp.*, *Burkholderia spp.*, *Bordetella spp.*, *Chlamydia spp.*, *Mycobacterium spp.*, *Prevotella spp.*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Streptococcus spp.* The assay also detects evolutionarily conserved regions of the 16S rRNA gene able to detect in a single array the broadest possible collection of bacteria involved in human biology (total 16s). For the microbiome profiling, the microbe's relative expression was compared to positive reference sample and normalized for an housekeeping gene (18s rRNA).

## **Pulmonary function measurement**

Pulmonary function tests, including spirometry, total lung capacity (assessed by means of the helium dilution technique), measurement of residual volume, and pulmonary diffusion capacity

(KCO), were performed using the Biomedin Spirometer, as previously described [9] according to published guidelines [10].

### **Quality of life assessment**

Health-related quality of life was measured at baseline and at the end the study by the total score on St. George's Respiratory Questionnaire (SGRQ). The scores range from 0 to 100, with lower scores indicating improvement. A linguistically valid translation of the SGRQ was used. The questionnaires were self-completed by the patients [11].

### **Definition of COPD exacerbation**

The exacerbation of COPD was defined as a symptomatic deterioration requiring treatment with antibiotic agents, systemic corticosteroids (moderate), hospitalization, or a combination of these (severe) [12].

### **Definition of chronic bronchitis**

Chronic bronchitis was defined as the presence of of cough and sputum production for at least 3 months in each of two consecutive years [1].

### **Sample size and Analyses**

The primary outcome was the assessment of bacterial load in the sputum of COPD patients after 12 months of treatment with SALM/FP compared with that in COPD patients treated with SALM. Secondary outcomes included assessment of viral detection, correlation between sputum

viral and/or bacterial load and clinical outcomes; and correlation between changes in sputum viral and/or bacterial load and sputum/systemic inflammatory cell profiles.

In a previous study [13], mean increase in bacterial load of  $0.46 \log_{10}$  colony-forming unit (CFU)/ml was observed over 1 yr in sputum samples of 30 stable COPD patients. The vast majority of these subjects (93%) were treated with an ICS containing medication. No prospective data on bacterial load in COPD patients not receiving ICS treatment is available. Based on these premises: i) we postulated no change in sputum bacterial load over 1 yr in COPD patients not receiving an ICS containing medications and ii) we calculated that the bacterial load would increase up to a mean value of about  $0.5 \log_{10}$  CFU ml<sup>-1</sup> in a COPD population treated (100%) with ICS containing medications. Given that, at variance with the population described in study by Wilkinson TM et al [13], we recruited only steroid naïve subjects, we considered that a greater increase in sputum bacterial load was to be expected in our study compared to the previous one [13], and a 20% increased value of  $0.6 \log_{10}$  CFU ml<sup>-1</sup> was assumed for our primary analysis. For the LABA (Salmeterol) treatment group no increase is assumed for the sample size calculation. The common standard deviation for the change is assumed to be 0.8. The Type-I error is set to 0.05 and the power should be at least 80%. Under these assumptions, a sample size of 30 patients per treatment group have to be available for the analysis. A similar number of subjects was enrolled in the study where increased sputum bacterial load was observed over 1 year [13].

Comparisons among groups were evaluated by unpaired t test or Mann-Whitney test or the  $\chi^2$  test, as appropriate. Changes at the end of the study versus baseline were evaluated by using paired t tests or Wilcoxon test, as appropriate. Multiple time-point comparisons within groups were assessed by means of ANOVA or the Kruskal–Wallis test, followed (when results were

significant) by using Student's t-tests or Mann–Whitney U tests, as appropriate. Correlation coefficients were calculated by using the Pearson test or nonparametric Spearman rank method, as appropriate. The evaluation and comparison of sputum microbial compositions were assessed by alpha (microbial diversity within a sample – Shannon's and Simpson's index) and beta diversity (microbial composition dissimilarity between samples - Bray-Curtis distances).

A multiple regression model was performed to evaluate the predictors of increased bacterial count in the study population. The change at 1-yr compared to baseline of the log<sub>10</sub> transformation of the total bacterial count was the dependent variable; the log<sub>10</sub> transformation of the bacterial counts at baseline, the treatment regimen (SALM/FP vs SALM), the smoking habit (former smoker vs active), the sputum eosinophil level at baseline ( $<$  or  $\geq 2\%$  of total non-squamous inflammatory cell count), and the interactions between variables, were considered as fixed effects.

A P-value of  $<0.05$  was considered significant. All analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) and SAS Advanced Analytics (Cary, NC 27513-2414, USA).



## RESULTS

### Patient characteristics

The start and end dates for the study were May 4, 2009, and May 31, 2013, respectively.

Consecutive COPD patients were screened for eligibility from May 4, 2009 to May 7, 2012 and recruited from May 27, 2009 to May 7, 2012 at the outpatient clinic of the Research Centre on Asthma and COPD, University of Ferrara, Italy.

No differences were found between the two groups of patients for the demographic characteristics. Similarly, no difference in the prevalence of comorbid conditions were found between treated groups. Arterial hypertension was the most frequent comorbid condition (40% and 37% in the SALM/FP vs LABA treated group respectively). Other comorbidities included: chronic ischemic heart disease (7% and 10% in the SALM/FP vs SALM treated group respectively), diabetes/metabolic syndrome (10% and 13% in the SALM/FP vs SALM treated group respectively) and chronic gastritis (13% and 7% in the SALM/FP vs SALM treated group respectively). No difference in the prevalence of comorbid conditions were found between treated groups. In our study population there was no difference between the two groups in the number of patients treated with statins (3 patients in the SALM/FP and 4 patients in the SALM group). A similar proportion of patients in the 2 groups suffered of diabetes at recruitment (7% and 10% in the SALM/FP vs SALM treated group respectively). None of them was treated with insulin, all of them received oral metformin. None of the patient was treated with immunosuppressant and/or chemotherapy or any other concomitant treatment with a pro-infective documented activity.

For ethical reasons we were not allow to perform high resolution CT scan of the chest for

screening purpose at recruitment. However, a CT scan, performed either in the year preceding the study for routine clinical characterization (GOLD) or following an acute episode, was available for 42 patients (70%) recruited to the study (19 in the SALM/FP groups and 23 in the SALM group). No radiologic signs of bronchiectasis were found in these patients. One patient performed CT scan during the study after a severe exacerbation without radiological evidence of bronchiectasis. We cannot exclude the presence of bronchiectasis in the remaining 18 patients that were equally distributed in the two groups (11 patients in the SALM/FP group and 7 patients in the SALM group). However, their clinical history of a smoking related progressive obstructive lung disease was not suggestive for a bronchiectatic component. Radiological signs (no quantification was performed) of centroacinar emphysema mainly distributed at the upper lobes were described in 15 (79%) and in 15 (70%) CT scans of patients randomised to SALM/FP and SALM group, respectively.

Twenty-eight % of patients experienced no exacerbations during the 1-yr study, 53% 1 exacerbation and 19%  $\geq 2$  exacerbations. The exacerbation rate reported in our study is similar to the exacerbation rate reported in the subgroup of moderate COPD patients with a similar post-bronchodilator FEV1 (of approximately 63% predicted) of the ECLIPSE study (0.85 exacerbation/patient/yr) [14].

### **Sputum sampling**

Overall 4 samples at baseline, 6 samples at 3 months, 4 samples at 6 months, 2 samples at 9 months and 6 samples at 1 year were repeated because sputum sampling was inadequate. As per the inclusion criteria, sputum samples were collected for all patients at baseline ( $n = 30$  in each group) and at the end of the 1-yr study treatment (for the primary analysis of the study). Sputum

was obtained in 90%, 80%, and 83% of patients treated with ICS/LABA and 86%, 73%, and 83% of patients treated with LABA alone at 3-, 6-, and 9-month study visits.

#### **Additional pulmonary function measurements**

The measurement of lung volumes (residual volume and total lung capacity) and diffusion capacity were performed for routine clinical characterization at recruitment or in the year preceding the study in 49 (82%) patients recruited to the study [26 (86%) in the SALM/FP group, 23 (77%) in the SALM group]. Overall, a similar increased residual volume ( $142.8 \pm 7.3$  and  $136.9 \pm 6.3\%$  predicted in SALM/FP and SALM group, respectively) and moderate reduction in diffusion capacity ( $KCO$   $67.1 \pm 4.8$  and  $59.5 \pm 6.1\%$  predicted in SALM/FP and SALM group, respectively) was found in both groups.

#### **Bacterial and viral load at stable state**

No correlations were found between demographic characteristics or functional parameters and bacterial load at baseline. Notably, the bacterial load at baseline was statistically higher in patients with chronic bronchitis compared to patients without chronic bronchitis ( $1.45 \times 10^8 \text{ Log}_{10} \text{ CFU/ml}$  vs  $2.53 \times 10^7 \text{ Log}_{10} \text{ CFU/ml}$ ;  $p < 0.01$ )

Atypical bacteria, were uncommon at stable state throughout the study; only one sample from the SALM/FP group contained *Chlamydia pneumoniae*, and one sample from the SALM alone group contained *Mycoplasma pneumoniae* (supplementary table S3).

The real-time PCR analysis found viral infections in 3.3%, 3.7%, 8.3%, 4%, and 3.3% of sputum samples of patients treated with SALM/FP and 3.3%, 3.8%, 0%, 8%, and 3.3% of sputum

samples in patients treated with SALM alone at baseline and after 3, 6, 9, and 12 months of treatment, respectively. Similar levels of total bacterial 16s were found at baseline in SALM/FP and SALM treated patients; overall an increase of total 16s was found in sputum of patients treated with SALM/FP but not SALM alone ( $0.97 \pm 0.12$  vs  $1.22 \pm 0.14$  and  $0.95 \pm 0.12$  vs  $0.93 \pm 0.13$  fold expression compare to positive control at baseline and at 1 yr in patients treated with SALM/FP ( $p=0.043$ ) or SALM alone ( $p=0.13$ ), respectively).

### **Clinical outcomes**

We observed no differences between the groups in FEV<sub>1</sub> absolute values at the end of the study and change versus baseline, with a post-bronchodilator FEV<sub>1</sub> decline over the 12 months by 48 mL in the SALM/FP group and by 45 mL in the SALM group (Fig. S2A). A significant improvement in health status, as assessed by changes in SGRQ scores, was found at the end of the study in the SALM/FP group ( $-2.67 \pm 1.1$  Unit) but not in the SALM group (Fig. S2B). A non-significant trend was found in the SALM/FP in the reduction of exacerbation rate compared with the previous year ( $0.73 \pm 0.13$  vs.  $0.86 \pm 0.13$  exacerbations/patient/yr during the study period vs. previous year respectively,  $p=0.093$ ). No change in the exacerbation frequency was observed in the SALM group ( $0.93 \pm 0.12$  vs.  $0.96 \pm 0.13$  exacerbations/patient/yr during the study period vs. previous year respectively). In the SALM/FP treated group a subset analysis was performed to evaluate the clinical outcomes in COPD patients with high ( $\geq 2\%$ ) or low ( $< 2\%$ ) blood eosinophil counts. No significant differences were found in FEV<sub>1</sub> decline and in the change of SGRQ score between the two subgroups of SALM/FP treated patient (supplementary table S5). In the SALM/FP treated

group, the number of exacerbation/patient/year was of  $0.81 \pm 0.13$  in patients with  $<2\%$  baseline sputum eosinophil and of  $0.64 \pm 0.14$  in the  $\geq 2\%$  baseline sputum eosinophils, with a numerical non-statistically significant ( $p=0.21$ ) reduction of 15% in the latter group.

One case of pneumonia occurred during the study, confirmed by chest X-ray, in a patient of the SALM group.

#### **Airway inflammation and microbiology during COPD exacerbations**

Sputum samples were available for all exacerbation events, and the collection was performed before the systemic treatments were started by the investigator physician. Overall, in line with previous studies [15], we found an increased airway total bacterial load and detection of PPB and virus at exacerbations compared with stable state, and this was paralleled by increased sputum inflammation. Total inflammatory cell counts significantly increased at exacerbation compared with the previous stable state visit in both groups (supplementary Fig. S3 panel A). Significant increases in neutrophil ( $p=0.008$ ) and eosinophil cell counts ( $p=0.031$ ) were found at exacerbation compared with the previous stable state visit in both groups. No difference was found in inflammatory cell counts between groups at exacerbations.

The total sputum bacteria load significantly increased at exacerbation compared with the previous stable state visit in both groups (supplementary Fig. S3 panel B).

In the SALM/FP group PPB were detected in 35% of the samples collected in stable conditions and in 64% of samples collected at exacerbation ( $p=0.0011$ ); in the SALM group PPB were detected in 34% and 60% of the samples obtained in stable state and during exacerbation, respectively ( $p=0.0011$  – supplementary table S3). In patients treated with SALM/FP respiratory viruses were detected in 4.4% of the samples collected in stable conditions and in 45% of

samples collected at exacerbation ( $p<0.0001$ ), whereas in the SALM group, respiratory viruses were detected in 3.8% and 46% of the samples obtained in stable state and during exacerbation, respectively ( $p<0.0001$ ).

#### **Predictors for changes in bacterial load in patients treated with SALM/FP combination**

The changes in bacterial load in the SALM/FP group did not correlate with any of the baseline demographic characteristics, functional parameters, and/or SGRQ values, nor with the changes of the clinical outcomes throughout the study. SALM/FP treatment lead to a similar increased bacterial load irrespective from the presence of chronic bronchitis ( $+8.4 \cdot 10^1 \log_{10}$  vs  $+2.1 \cdot 10^1 \log_{10}$  fold changes in patients with or without chronic bronchitis, respectively –  $p=0.67$ ). In the SALM/FP group no difference was found in the increase bacterial load between patients receiving at least one course of antibiotics because of an exacerbation ( $n=20$ ) compared to patients who did not receive any antibiotic course ( $n=10$ ) ( $1.1 \pm 0.78$  vs  $1.85 \pm 0.31 \log_{10}$  change in total bacterial load,  $p>0.05$  respectively). No difference was found in the sputum microbial composition between patients experiencing increase vs decrease/no change in total bacterial load after 1-yr of treatment (data not shown).

When patients with blood eosinophil levels  $\geq 2\%$  were considered, we found that after 1-yr treatment with SALM/FP there was an increased bacterial load vs baseline in 50% (7 out of 14) subjects, a reduced bacterial load in 29% (4 out of 14) and no change in the remaining 3 patients (Fig. 4A). No difference was found in demographic or lung function parameter among patients with blood eosinophil levels  $\geq 2\%$  between those with an increased or decreased bacterial load over 1-yr treatment with SALM/FP. In this subgroup of patients, the trend of bacterial load (increase vs decrease) was found to be related with bacterial load level at baseline: significant

higher bacterial load levels at baseline was found in patients with a decrease bacterial load over time compared to the others ( $10^5$  [ $10^3 - 10^6$ ] CFU/ml vs  $10^8$  [ $10^5 - 10^9$ ] CFU/ml, respectively;  $p=0.01$ ) (Fig. 4A). Interestingly, in patients with blood eosinophil levels  $<2\%$  the SALM/FP treatment resulted in increased bacterial load (92% of patients - 11 out of 12) irrespective from baseline bacterial load. By multiple regression analysis we confirmed that: i) the total bacterial count at baseline (as expressed as log<sub>10</sub>); ii) the treatment regimen and iii) the interaction between treatment and sputum eosinophil levels at baseline were predictors of increase bacterial count at the end of the study. In particular, we confirmed that only the interaction between SALM/FP treatment and low ( $<2\%$ ) eosinophil counts resulted in significant increased bacterial load at the end of the study (Table S9).

325 Supplementary Table S1. List of the pathogens detectable by RespiFinder assay.

<b>VIRUS</b>	<b>NUCLEIC ACID</b>
Adenovirus	RNA
Bocavirus	RNA
Corona 229E	RNA
Corona HKU1	RNA
Corona NL63	RNA
Corona OC43	RNA
hMPV	RNA
Influenza A	RNA
Influenza B	RNA
Influenza A H1N1v	RNA
Parainfluenza 1	RNA
Parainfluenza 2	RNA
Parainfluenza 3	RNA
Parainfluenza 4	RNA
Rhinovirus/Enterovirus	DNA
RSV-A	DNA
RSV-B	DNA
<b>BACTERIA</b>	<b>NUCLEIC ACID</b>
<i>Bordetella pertussis</i>	DNA
<i>Chlamydomypha pneumoniae</i>	DNA
<i>Legionella pneumophila</i>	DNA
<i>Mycoplasma pneumoniae</i>	DNA

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333 Supplementary Table S2. List of pathogens (16s RNA sequences) detectable by Microbial DNA qPCR array.

Phylum	Species
Actinobacteria	<i>Corynebacterium diphtheriae</i>
	<i>Mycobacterium avium</i>
	<i>Mycobacterium kansasii</i>
	<i>Mycobacterium tuberculosis</i> ; <i>Mycobacterium bovis</i> ; <i>Mycobacterium africanum</i>
	<i>Nocardia cyriacigeorgica</i> ; <i>Nocardia abscessus</i> ; <i>Nocardia cummidelens</i> ; <i>Nocardia flavorosea</i> ; <i>Nocardia pseudobrasiliensis</i>
	<i>Rhodococcus equi</i>
Bacteroidetes	<i>Prevotella bivia</i>
	<i>Prevotella oris</i>
Chlamydiae	<i>Chlamydia trachomatis</i>
	<i>Chlamydophila pneumoniae</i>
	<i>Chlamydophila psittaci</i>
Firmicutes	<i>Bacillus anthracis</i>
	<i>Clostridium sordellii</i>
	<i>Peptostreptococcus anaerobius</i>
	<i>Staphylococcus aureus</i>
	<i>Streptococcus agalactiae</i>
	<i>Streptococcus pneumoniae</i>
	<i>Streptococcus pyogenes</i>
Fusobacteria	<i>Streptobacillus moniliformis</i>
Proteobacteria	<i>Acinetobacter baumannii</i>
	<i>Acinetobacter rhizosphaerae</i> ; <i>Acinetobacter calcoaceticus</i>
	<i>Actinobacillus hominis</i>
	<i>Bordetella bronchiseptica</i> ; <i>Bordetella pertussis</i> ; <i>Bordetella parapertussis</i>
	<i>Burkholderia vietnamiensis</i> ; <i>Burkholderia pyrrocinia</i> ; <i>Burkholderia cenocepacia</i> ; <i>Burkholderia cepacia</i>
	<i>Burkholderia gladioli</i>
	<i>Burkholderia pseudomallei</i> ; <i>Burkholderia mallei</i>
	<i>Coxiella burnetii</i>
	<i>Francisella novicida</i> ; <i>Francisella tularensis</i>
	<i>Haemophilus influenzae</i>
	<i>Legionella pneumophila</i>
	<i>Moraxella catarrhalis</i>
	<i>Neisseria meningitidis</i>
	<i>Proteus vulgaris</i> ; <i>Proteus mirabilis</i>
	<i>Pseudomonas aeruginosa</i>
	<i>Pseudomonas geniculata</i> ; <i>Stenotrophomonas maltophilia</i> ; <i>Xanthomonas retroflexus</i>
	<i>Yersinia pseudotuberculosis</i> ; <i>Yersinia pestis</i>
Tenericutes	<i>Mycoplasma pneumoniae</i>
Mycetes	<i>Aspergillus flavus</i>
	<i>Aspergillus fumigatus</i>
	<i>Candida</i>
	<i>Pneumocystis jirovecii</i>

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	V0	V1	V2	V3	V4	E
SALM/FP treated group	<p><b>PPB:</b></p> <ul style="list-style-type: none"> <li>- S. aureus: 1</li> <li>- Moraxella catarrhalis: 1</li> <li>- H. influenzae: 2</li> <li>- E. coli: 1</li> <li>- Stenotroph. maltophilia: 1</li> <li>- H. parainfluenzae: 2</li> <li>- S. pneumoniae: 1</li> <li>- Serratia marcescens: 1</li> <li>- P. aeruginosa: 1</li> </ul> <p><b>APB:</b> 0</p> <p><b>Virus:</b></p> <ul style="list-style-type: none"> <li>- Adenovirus: 1</li> </ul> <p><b>Detection PPB: 30%</b> <b>Detection APB: 0%</b> <b>Detection Virus: 3.3%</b></p>	<p><b>PPB:</b></p> <ul style="list-style-type: none"> <li>- S. aureus: 1</li> <li>- H. influenzae: 3</li> <li>- E. coli: 1</li> <li>- H. parainfluenzae: 4</li> <li>- S. pneumoniae: 2</li> <li>- P. aeruginosa: 2</li> </ul> <p><b>APB:</b></p> <ul style="list-style-type: none"> <li>- Chlamydothyla P.: 1</li> </ul> <p><b>Virus:</b></p> <ul style="list-style-type: none"> <li>- Parainfluenza 1: 1</li> </ul> <p><b>Detection PPB: 37%</b> <b>Detection APB: 3.7%</b> <b>Detection Virus: 3.7%</b></p>	<p><b>PPB:</b></p> <ul style="list-style-type: none"> <li>- S. aureus: 1</li> <li>- Moraxella catarrhalis: 1</li> <li>- H. influenzae: 1</li> <li>- Kleb. oxytoca: 1</li> <li>- E. cloacae: 1</li> <li>- S. pneumoniae: 2</li> <li>- Serratia marcescens: 1</li> <li>- P. aeruginosa: 2</li> <li>- H. parainfluenzae: 1</li> </ul> <p><b>APB:</b> 0</p> <p><b>Virus:</b></p> <ul style="list-style-type: none"> <li>- hMPV: 1</li> <li>- Rhinovirus/Enterovirus: 1</li> </ul> <p><b>Detection PPB: 38%</b> <b>Detection APB: 0%</b> <b>Detection Virus: 8.3%</b></p>	<p><b>PPB:</b></p> <ul style="list-style-type: none"> <li>- S. aureus: 2</li> <li>- Moraxella catarrhalis: 1</li> <li>- H. influenzae: 5</li> <li>- S. pneumoniae: 3</li> <li>- P. aeruginosa: 1</li> <li>- H. parainfluenzae: 1</li> </ul> <p><b>APB:</b> 0</p> <p><b>Virus:</b></p> <ul style="list-style-type: none"> <li>- Influenza A: 1</li> </ul> <p><b>Detection PPB: 36%</b> <b>Detection APB: 0%</b> <b>Detection Virus: 4%</b></p>	<p><b>PPB:</b></p> <ul style="list-style-type: none"> <li>- S. aureus: 1</li> <li>- Moraxella catarrhalis: 2</li> <li>- H. influenzae: 2</li> <li>- S. pneumoniae: 3</li> <li>- Serratia marcescens: 1</li> <li>- H. parainfluenzae: 1</li> <li>- Stenotroph. maltophilia: 1</li> <li>- P. aeruginosa: 1</li> </ul> <p><b>APB:</b> 0</p> <p><b>Virus:</b></p> <ul style="list-style-type: none"> <li>- RSV-A: 1</li> </ul> <p><b>Detection PPB: 33%</b> <b>Detection APB: 0%</b> <b>Detection Virus: 3.3%</b></p>	<p><b>PPB:</b></p> <ul style="list-style-type: none"> <li>- S. epidermidis: 2</li> <li>- S. aureus: 1</li> <li>- E. coli: 1</li> <li>- H. influenzae: 2</li> <li>- S. pneumoniae: 2</li> <li>- Moraxella catarrhalis: 2</li> <li>- H. parainfluenzae: 3</li> <li>- P. aeruginosa: 1</li> </ul> <p><b>APB:</b></p> <ul style="list-style-type: none"> <li>- Legionella p.: 1</li> <li>- Mycoplasma p.: 1</li> </ul> <p><b>Virus:</b></p> <ul style="list-style-type: none"> <li>- Adenovirus: 3</li> <li>- hMPV: 2</li> <li>- Influenza A: 2</li> <li>- Parainfluenza 1: 2</li> <li>- Parainfluenza 2: 2</li> <li>- Parainfluenza 3: 1</li> <li>- Rhinovirus/Enterovirus: 5</li> </ul> <p><b>Detection PPB: 64%</b> <b>Detection APB: 9%</b> <b>Detection Virus: 45%</b></p>
SALM treated group	<p><b>PPB:</b></p> <ul style="list-style-type: none"> <li>- Klebsiella P.: 1</li> <li>- H. influenzae: 2</li> <li>- E. coli: 3</li> <li>- S. aureus: 3</li> <li>- S. pneumoniae: 2</li> <li>- Moraxella catarrhalis: 1</li> </ul> <p><b>APB:</b> 0</p> <p><b>Virus:</b></p> <ul style="list-style-type: none"> <li>- hMPV: 1</li> </ul> <p><b>Detection PPB: 33%</b> <b>Detection AB: 0%</b> <b>Detection Virus: 3.3%</b></p>	<p><b>PPB:</b></p> <ul style="list-style-type: none"> <li>- P. aeruginosa: 1</li> <li>- Kleb. Oxytoca: 1</li> <li>- H. influenzae: 3</li> <li>- E. coli: 1</li> <li>- H. parainfluenzae: 1</li> <li>- S. aureus: 1</li> <li>- Neisseria Sicca: 1</li> <li>- Moraxella catarrhalis: 1</li> </ul> <p><b>APB:</b> 0</p> <p><b>Virus:</b></p> <ul style="list-style-type: none"> <li>- Parainfluenza 1: 1</li> </ul> <p><b>Detection PPB: 35%</b> <b>Detection AB: 0%</b> <b>Detection Virus: 3.8%</b></p>	<p><b>PPB:</b></p> <ul style="list-style-type: none"> <li>- S. pneumoniae: 2</li> <li>- H. influenzae: 4</li> <li>- E. coli: 2</li> <li>- S. aureus: 1</li> </ul> <p><b>APB:</b></p> <ul style="list-style-type: none"> <li>- Mycoplasma p.: 1</li> </ul> <p><b>Virus:</b> 0</p> <p><b>Detection PPB: 32%</b> <b>Detection AB: 4.5%</b> <b>Detection Virus: 0%</b></p>	<p><b>PPB:</b></p> <ul style="list-style-type: none"> <li>- S. pneumoniae: 1</li> <li>- H. influenzae: 3</li> <li>- E. Cloacae: 1</li> <li>- S. aureus: 1</li> <li>- Candida Albicans: 2</li> <li>- P. aeruginosa: 1</li> <li>- Gardnerella Vaginalis: 1</li> <li>- E. Coli: 1</li> <li>- Citrobacter Diversus: 1</li> </ul> <p><b>APB:</b> 0</p> <p><b>Virus:</b></p> <ul style="list-style-type: none"> <li>- Adenovirus: 1</li> <li>- Rhinovirus/Enterovirus: 1</li> </ul> <p><b>Detection PPB: 36%</b> <b>Detection AB: 0%</b> <b>Detection Virus: 8%</b></p>	<p><b>PPB:</b></p> <ul style="list-style-type: none"> <li>- S. pneumoniae: 2</li> <li>- H. influenzae: 4</li> <li>- H. parainfluenzae: 1</li> <li>- S. aureus: 1</li> <li>- Klebsiella P.: 1</li> <li>- P. aeruginosa: 1</li> <li>- Moraxella catarrhalis: 1</li> </ul> <p><b>APB:</b> 0</p> <p><b>Virus:</b></p> <ul style="list-style-type: none"> <li>- Rhinovirus/Enterovirus: 1</li> </ul> <p><b>Detection PPB: 33%</b> <b>Detection AB: 0%</b> <b>Detection Virus: 3.3%</b></p>	<p><b>PPB:</b></p> <ul style="list-style-type: none"> <li>- S. aureus: 2</li> <li>- H. influenzae: 5</li> <li>- S. pneumoniae: 5</li> <li>- Moraxella catarrhalis: 2</li> <li>- H. parainfluenzae: 1</li> <li>- P. aeruginosa: 2</li> </ul> <p><b>APB:</b></p> <ul style="list-style-type: none"> <li>- Mycoplasma pneum.: 1</li> </ul> <p><b>Virus:</b></p> <ul style="list-style-type: none"> <li>- Adenovirus: 3</li> <li>- hMPV: 5</li> <li>- Influenza A: 3</li> <li>- Parainfluenza 1: 3</li> <li>- Rhinovirus/Enterovirus: 5</li> </ul> <p><b>Detection PPB: 60%</b> <b>Detection APB: 3.5%</b> <b>Detection Virus: 46%</b></p>

### Supplementary table S3

List of airway pathogenic microorganisms detected at stable state and at COPD exacerbations, in the salmeterol/fluticasone propionate (SALM/FP) and Salmeterol (SALM) treated groups. PPB: potentially pathogenic bacteria. APB: atypical pathogenic bacteria. V0: baseline visit, V1: visit after 3-month of treatment, V2: visit after 6-month of treatment, V3: visit after 9-month of treatment and V4: visit after 12-month of treatment. E: exacerbation.

Supplementary table S4. Sputum differential cell counts [median (min –max)].

	<b>Salmeterol/Fluticasone Propionate treated group (SALM/FP)</b>				
	<b>V0 (baseline)</b>	<b>V1 (3 months)</b>	<b>V2 (6 months)</b>	<b>V3 (9 months)</b>	<b>V4 (12 months)</b>
<b>Total cell count</b> (10 <sup>5</sup> cells/mL)	8.8 [1 – 35.0]	7.5 [1 – 43.4]	4.9 [0.7 – 49]	7.1 [1.3 – 42]	6.5 [1.2 – 30.8]
<b>Macrophages (%)</b>	18.6 [1.5 – 50]	17.5 [0 - 44]	12.3 [0 - 47]	11 [0 - 38.5]	13.8 [0 - 55]
<b>Neutrophils (%)</b>	79.5 [47 – 98.5]	80 [52 – 99]	86 [53 – 98]	89 [59 – 99]	85 [45 – 99]
<b>Eosinophils (%)</b>	1 [0 – 6]	1 [0 - 6]	1 [0 – 3.5]	1 [0 – 3]	1 [0 – 3]
<b>Lymphocytes (%)</b>	0 [0 – 1]	0 [0 – 1]	0 [0 – 1]	0 [0 – 1]	0 [0 – 1]
	<b>Salmeterol treated group (SALM)</b>				
	<b>V0 (baseline)</b>	<b>V1 (3 months)</b>	<b>V2 (6 months)</b>	<b>V3 (9 months)</b>	<b>V4 (12 months)</b>
<b>Total cell count</b> (10 <sup>5</sup> cells/mL)	5.7 [1.1 – 34.6]	5.4 [1.3 – 23]	4.75 [1.3 – 23]	5.4 [1.2 – 39]	6.1 [1.2 – 38.8]
<b>Macrophages (%)</b>	12 [0 – 39]	17.8 [0 -41]	14 [0.5 – 46.5]	9.5 [0 - 31]	12.5 [0 - 47.5]
<b>Neutrophils (%)</b>	87 [61 – 100]	81 [57 - 100]	85 [53.5 – 99]	89 [66 – 99]	87.25 [51-100]
<b>Eosinophils (%)</b>	0.5 [0 – 4]	0.5 [0 – 3]	0.5 [0 – 5.5]	0.5 [0 – 3]	1 [0 – 4]
<b>Lymphocytes (%)</b>	0 [0 – 1]	0 [0 – 1.5]	0 [0 – 1]	0 [0 – 1]	0 [0 – 1.5]

Supplementary table S5: *Clinical outcomes in SALM/FP treated group according to blood eosinophil levels.*

<b>SALM/FP treated group</b>	<b>Patients with blood eosinophil counts <math>\geq 2\%</math> (n=14)</b>	<b>Patients with blood eosinophil counts <math>&lt; 2\%</math> (n=12)</b>
Post-FEV1 decline (ml)	-47 $\pm$ 46 ml	-56 $\pm$ 54 ml
Change in SGRQ (units)	-2.50 $\pm$ 0.68	-1.88 $\pm$ 1.72

Supplementary Table S6: microbial composition dissimilarity between groups assessed by beta-diversity- Bray-Curtis distances

Test for homogeneity of multivariate dispersions	F test
Treatment difference at Baseline	
p-value	0.37862
Treatment difference after one year	
p-value	0.65174

Supplementary Table S7 microbial  $\alpha$  diversity in sputum samples evaluated by Shannon's index

	SALM/FP N=30	SALM N=30
Baseline		
n	30	30
Mean (SD)	1.765 (0.357)	1.841 (0.369)
Median	1.792	1.946
Range	1.10 ; 2.40	0.69 ; 2.30
1-vr		
n	30	30
Mean (SD)	1.983 (0.325)	1.739 (0.596)
Median	2.079	1.869
Range	1.10 ; 2.40	0.00 ; 2.40
Change from baseline		
n	30	30
Mean (SD)	0.219 (0.517)	-0.102 (0.619)
Median	0.288	0.000
Range	-0.79 ; 1.20	-1.95 ; 0.79
Paired t-test, p-value	0.028	0.373

Supplementary Table S8 microbial  $\alpha$  diversity in sputum samples evaluated by Simpson's index

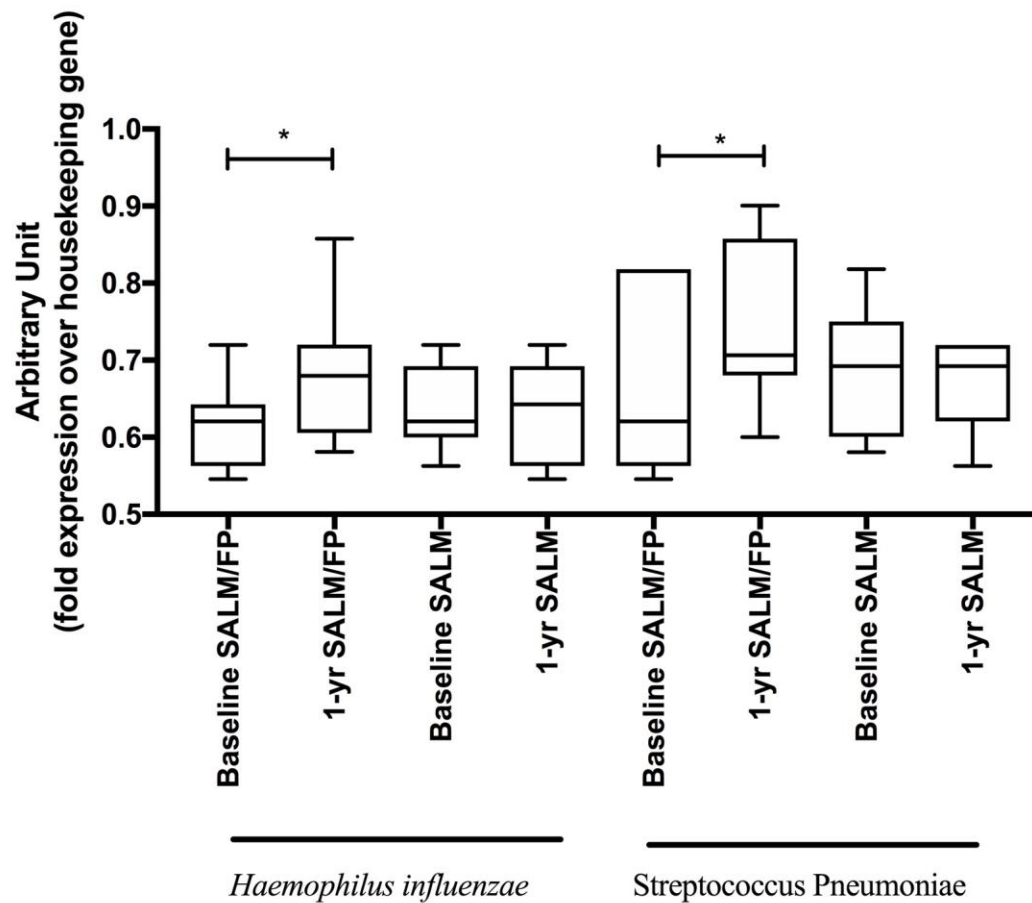
	SALM/FP N=30	SALM N=30
Baseline		
n	30	30
Mean (SD)	0.818 (0.068)	0.828 (0.084)
Median	0.833	0.857
Range	0.67 ; 0.91	0.50 ; 0.90
1-yr		
n	30	30
Mean (SD)	0.854 (0.059)	0.777 (0.222)
Median	0.875	0.845
Range	0.67 ; 0.91	0.00 ; 0.91
Change from baseline		
n	30	30
Mean (SD)	0.036 (0.094)	-0.051 (0.228)
Median	0.042	0.000
Range	-0.17 ; 0.23	-0.86 ; 0.17
Paired t-test, p-value	0.042	0.229



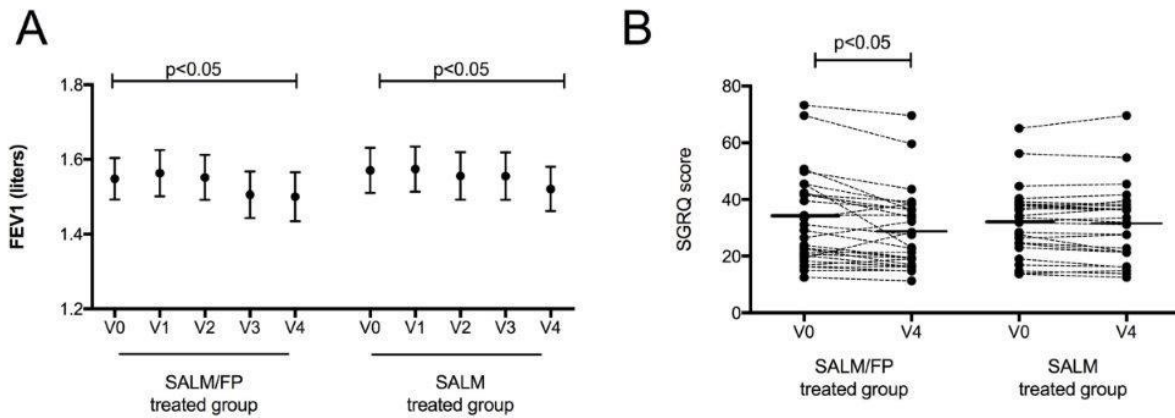
**Supplementary table S9.** multiple regression model to evaluate the predictors of increased bacterial count in the study population

Number of patients			
SALM/FP	30		
SALM	30		
Number of patients considered in the model			
SALM/FP	30		
SALM	30		
p-values for fixed effects			
Log10 Baseline bacterial count	<0.001		
Treatment	<0.001		
Smoking status	0.260		
Sputum Eosinophil levels at baseline	0.360		
Treatment and Sputum Eosinophil levels at baseline	0.039		
	Log 10 ratio of total bacterial count at 1-yr	95% confidence interval	p-value
Estimated differences			
SALM/FP vs SALM	1.822	[1.047 - 2.597]	<0.001
EOS <2% vs EOS ≥2% in SALM/FP	1.193	[0.077 - 2.309]	0.037
EOS <2% vs EOS ≥2% in SALM	-0.467	[-1.581 - 0.648]	0.405
SALM/FP vs SALM in EOS <2%	2.652	[1.689 - 3.614]	<0.001
SALM/FP vs SALM in EOS ≥2%	0.992	[-0.239 - 2.224]	0.112

**Supplementary Fig. S1.** Bacterial load at baseline and after 1 year of treatment for *Haemophilus influenzae* and *Streptococcus pneumoniae* by Real Time qPCR in salmeterol/fluticasone (SALM/FP) and salmeterol alone (SALM) treated COPD patients (\* $p < 0.05$ ).

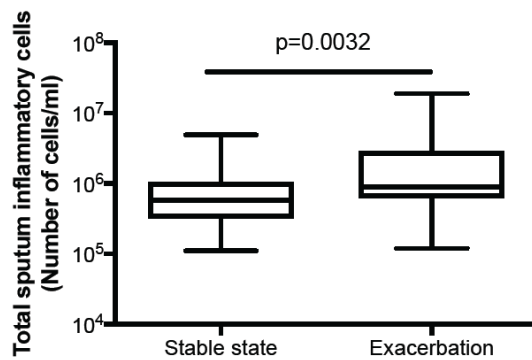


**Supplementary Fig. S2** Clinical outcomes. Panel A) Lung function in COPD patients in the Salmeterol/Fluticasone propionate (SALM/FP) and Salmeterol (SALM) groups. Panel B) Saint George Respiratory Questionnaire (SGRQ) total scores. (V0, baseline; V1, 3 months; V2, 6 months; V3, 9 months; V4, 12 months).

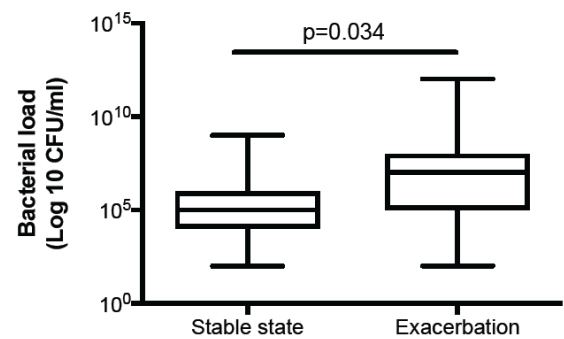


**Supplementary Fig S3** COPD exacerbations. Panel A) Total inflammatory cell counts in sputum at exacerbation and at the previous stable state visit. Panel B) Total airway bacterial load at exacerbation and at the previous stable state visit.

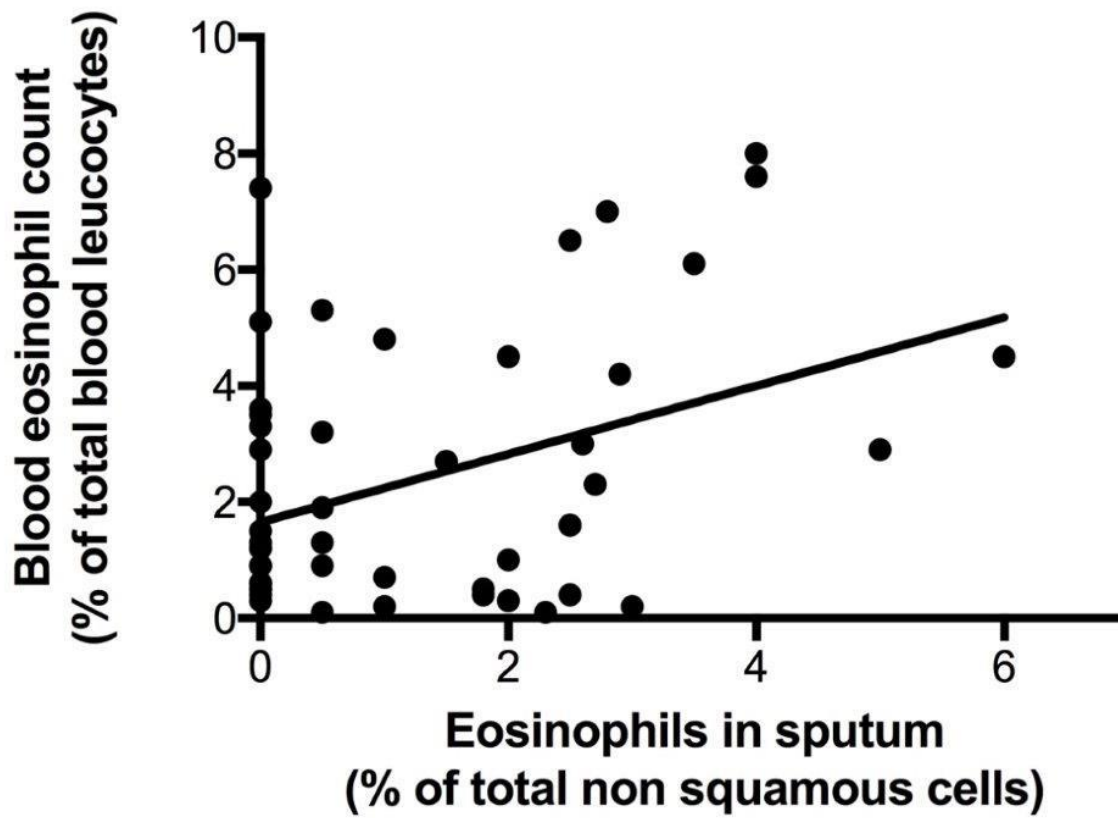
**A**



**B**



**Supplementary Fig S4.** Correlation between blood and sputum eosinophil counts at baseline.



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