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Variability and effects of bronchial colonisation in patients with moderate COPD

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ABSTRACT: Sputum and lung function were periodically assessed in stable moderate chronic obstructive pulmonary disease (COPD) outpatients to determine relationships between bronchial colonisation and inflammation.

Relationships between potentially pathogenic microorganism (PPM) typology, bronchial inflammation (neutrophilia, tumour necrosis factor-α, interleukin (IL)-1β, IL-6, IL-8, IL-10 and IL-12) and post-bronchodilator decline in forced expiratory volume in 1 s (FEV_1) were analysed. PPMs periodically showing the same molecular profile using pulse field gel electrophoresis were considered long-term persistent.

Bronchial colonisation was observed in 56 out of 79 follow-up examinations (70.9%) and was mainly due to *Haemophilus influenzae*, *Pseudomonas aeruginosa* and enterobacteria (n=47). These PPMs were all related to sputum neutrophilia ($p \leqslant 0.05$, Chi-squared test), and *H. influenzae* was related to higher levels of IL-1β ($p=0.005$) and IL-12 ($p=0.01$), with a dose–response relationship (Spearman's correlation coefficient of 0.38 for IL-1β ($p=0.001$), and of 0.32 for IL-12 ($p=0.006$)). *Haemophilus parainfluenzae* was not associated with an identifiable inflammatory response. Long-term persistence of the same strain was observed in 12 examinations (21.4%), mainly due to *P. aeruginosa* or enterobacteria. A neutrophilic bronchial inflammatory response was associated with a statistically significant decline in FEV_1 during follow-up (OR 2.67, 95% CI 1.07–6.62).

A load-related relationship to bronchial inflammation in moderate COPD was observed for colonisation by *H. influenzae*, but not for colonisation by *H. parainfluenzae*.

KEYWORDS: Bronchial colonisation, chronic obstructive pulmonary disease, *Haemophilus influenzae*, interleukin-1β, interleukin-12, potentially pathogenic microorganisms

The bronchial tree and the pulmonary parenchyma are sterile in healthy subjects, but in patients with chronic obstructive pulmonary disease (COPD) potentially pathogenic microorganisms (PPMs) are often recovered from bronchial secretions during periods of clinical stability and, particularly, during episodes of exacerbation, when PPM loads increase significantly [1, 2]. Most studies on PPM colonisation in COPD have focused on patients with severe disease [3–5]. In contrast, the relationships between the microbiology of bronchial colonisation and local inflammatory response in moderate COPD patients, when therapeutic interventions are expected to have maximal effects, have only been assessed in small selected population samples [6–8]. With the hypothesis that the characteristics of bronchial colonisation have an effect on bronchial inflammation and lung function that may be identified in stable outpatients with moderate COPD, the present study sought: first, to determine the prevalence and load of bronchial colonisation in a cohort of COPD outpatients with moderate airflow limitation never admitted to a hospital for an exacerbation of the disease; and secondly, to investigate in these patients the relationships between colonisation characteristics, bronchial inflammation markers and post-bronchodilator changes in forced expiratory volume in 1 s (FEV_1) during follow-up.

METHODS

Design and study population

We enrolled a cohort of stable COPD outpatients diagnosed according to the criteria of the Global Initiative for Chronic Obstructive Lung Disease (GOLD) [9] and reporting no admissions for respiratory symptoms. Patients were examined at

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baseline in stable condition from ⩾8 weeks, and their sociodemographic data, smoking habits, respiratory symptoms, treatments, sputum microbiological and inflammatory characteristics, and lung function were recorded. 9 months (interval 7–11 months) after this baseline assessment, a follow-up visit was scheduled at a time when the patient had been in a stable condition for ⩾8 weeks. That visit included a second assessment of sputum characteristics, together with recording of exacerbation history and assessment of lung function changes since the previous visit. Patients were excluded if they: were <40 yrs of age; had ever been admitted to a hospital for respiratory symptoms; were diagnosed with asthma, cystic fibrosis, neoplasia or bronchiectasis, and/or were being treated with oral corticosteroids or immunosuppressors for any reason. Additionally, patients who did not maintain the same smoking habits and baseline bronchodilator and inhaled corticosteroid treatment throughout the follow-up interval were excluded from analysis. Acute episodes of increased dyspnoea, sputum production and/or purulence appearing during follow-up and treated with antibiotics and/or oral corticosteroids by a physician were considered as exacerbations [10, 11]. The present study was reviewed and approved by the local research ethics committee in Catalonia, Spain.

Interview questionnaire and lung function

The interview questionnaire, which included items on age, sex and chronic bronchitis (defined as cough and phlegm >3 months each yr) was obtained from the European Community Respiratory Health Survey (ECRHS) [12, 13]. Current treatments and previous exacerbations were also recorded. All subjects performed forced spirometry and reversibility tests in the morning with the same dry rolling-seal spirometer (Spirometrics, Gray, ME, USA) at baseline and follow-up visits according to standard techniques [14]. Forced vital capacity and FEV_1 were measured and were compared with age- and height-adjusted reference values obtained from selected volunteers from the Barcelona province [15]. This was followed by a reversibility test with salbutamol. Results were expressed as absolute values (mL) and percentages of the reference values.

Sputum sampling and analysis

An induced sputum sample was obtained and processed within 60 min at each visit according to standard methods [16, 17]. In brief, the patient was pre-treated with an inhaled β_2-agonist 10 min before nebulisation of isotonic saline (0.9%); this was followed by increasing concentrations of hypertonic saline (3, 4 and 5%) for 7 min with each concentration. After each induction the patient attempted to obtain a sputum sample by coughing, and the nebulisation procedure was halted when the sputum volume collected was ⩾1 mL [18]. Sputum induction was performed after 6 h of abstinence in current smokers. Samples with >25 leukocytes per field (Murray-Washington ⩾3) were considered indicative of a neutrophilic inflammatory response [19, 20].

Microbiological determinants

The sputum sample was weighed and processed with an equal volume of dithiothreitol (Sputasol; Oxoid Ltd, Basingstoke, UK), cultured, and the microbial load of the sample was determined [21]. The determination of microbial typology and load was carried out by means of serial dilutions and culture in selective media, according to standard methods [22], with quantitative cultures expressed as colony-forming units (cfu)·mL^{-1}. For the purposes of this study, cultures were considered positive for bronchial colonisation according to previously defined criteria [23–25] if they grew microorganisms, at loads of ⩾100 cfu·mL^{-1}, that were considered as potentially pathogenic, such as *Haemophilus influenzae, Haemophilus parainfluenzae, Streptococcus pneumoniae, Moraxella catarrhalis, Pseudomonas aeruginosa*, enterobacteria and/or *Staphylococcus aureus*.

Molecular genotyping

Molecular PPM typing was performed using pulse field gel electrophoresis (PFGE) to determine whether a PPM recovered from consecutive samples corresponded to the persistence of the same strain or to the acquisition of a new one [26]. In brief, chromosomal DNA from multiple (more than four) PPMs growing in chocolate agar was extracted with agarose and incubated at 37°C for 18 h. After enzyme digestion with Sma I (New England Biolabs, Ipswich, MA, USA), restriction fragments were separated in agarose gel with tris-borate-ethylenediaminetetraacetic acid (Sigma Chemical, St Louis, MI, USA) through PFGE using a homogeneous electric camp contour clamp (CHEF DR II system; BioRad, Ivey sur Seine, France), beginning with an initial 5.6-s pulse that was linearly increased until a 40.6-s pulse was reached and then maintained for 24 h at 5 V·cm^{-1} and 14°C. Bacteriophage λ concatemer (New England Biolabs) was included as the molecular weight DNA marker. Obtained patterns were photographed with a 360-nm transilluminator after staining, and analysed using Diversity Database Software (BioRad). PPMs cultured from follow-up sputum samples obtained from patients who grew the same species at baseline and showed the same molecular profile in both samples analysed by PFGE were considered long-term persistent strains, whereas PPMs cultured at follow-up which were not present in the baseline sample were considered as strains acquired during follow-up (fig. 1).

Inflammatory markers

The remaining sputum was centrifuged and the concentrations of several cytokines (tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, IL-10 and IL-12) were measured in the supernatant using a cytokine bead array (BD Biosciences, San Diego, CA, USA). This assay system consists of a mixture of uniform bead types that contain different fluorescence intensities of a red-emitting dye. A capture antibody against each cytokine is covalently coupled to a type of bead, and cytokines bound to these antibodies are detected by use of phycoerythrin-labelled antibodies. The fluorescence intensity measured with phycoerythrin is proportional to the cytokine concentration in the sample and is quantified for every cytokine from a calibration curve. The detection limits of these assays were 3.7 pg·mL^{-1} for TNF-α, 7.2 pg·mL^{-1} for IL-1β, 2.5 pg·mL^{-1} for IL-6, 3.6 pg·mL^{-1} for IL-8, 3.3 pg·mL^{-1} for IL-10 and 1.8 pg·mL^{-1} for IL-12.

Statistical analysis

All data were added to a database and analysed using the SPSS statistical software package version 15 (Chicago, IL, USA). Results are expressed as absolute and relative frequencies for

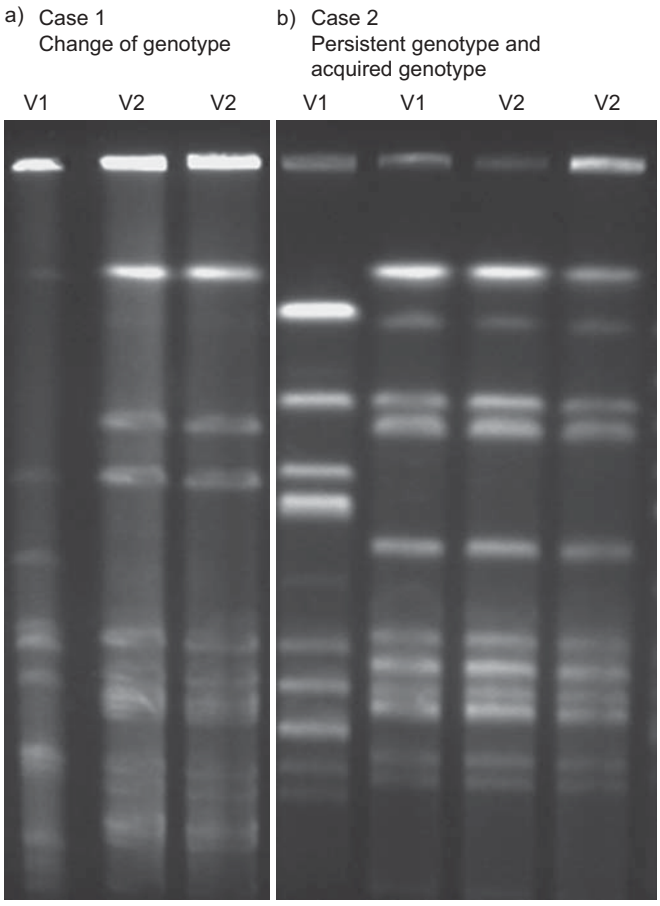

FIGURE 1. Pulse-field electrophoresis of samples growing *Haemophilus influenzae* in baseline and follow-up samples. a) Persistent *H. influenzae* strain, and b) appearance of a strain newly acquired during follow-up.

categoric variables and as means±SD, or median (interquartile range (IQR)) for continuous variables, as appropriate depending on the distribution of data. A value of half the lower detection limit was used for all measures of continuous variables showing a result below that value.

Descriptive statistics were compiled for sociodemographic characteristics and all the examinations were performed at baseline and at the end of follow-up. Sputum characteristics recorded at both visits were microbial profile and load, neutrophilia according to Murray-Washington criteria (⩾3), and concentration of cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12). Additional follow-up measurements were length of time in months between visits, appearance and frequency of exacerbations (calculated as number of exacerbations in the period/number of months of follow-up × 12); and change in the post-bronchodilator FEV1 in mL during the follow-up period (calculated as follow-up FEV1 - previous FEV1/number of months of follow-up × 12).

Analysis of the relationships between microbial typology and bronchial inflammation, measured as sputum neutrophilia and cytokine concentration, were performed at the end of follow-up (Chi-squared, Fisher's exact and Mann–Whitney U-tests). In this analysis, PPMs cultured from follow-up sputum samples and showing the same molecular profile as PPMs recovered at baseline were considered as long-term persistent strains, whereas PPMs cultured at follow-up but not present at baseline were considered as acquired. First, correlations between microbial load and bronchial inflammation parameters (Mann–Whitney U-test and the Spearman's correlation coefficient) were calculated. Next, the differences between colonised patients who harboured long-term persistent PPMs and microorganisms acquired during follow-up were analysed, through the assessment of the relationships between long-term microbial persistence and microbial profile, load, sputum neutrophilia and concentration of cytokines at the follow-up visit. Finally, the impact of bronchial inflammation on the change in post-bronchodilator FEV1 during the follow-up period was assessed using logistic regression modelling, considering an FEV1 decline greater than the median decline as the outcome, and age, current smoking, use of inhaled corticosteroids and appearance of an exacerbation during follow-up as covariates. The results of these analyses were expressed as crude and adjusted OR with 95% CI. Multivariate models were adjusted for smoking and other covariates showing an association with the outcome variable in the univariate models ($p<0.20$). All statistical tests were two sided, and a p-value of ⩾0.05 less was reported as statistically significant.

TABLE 1 Sociodemographic characteristics

Patients n	40
Age yrs	66.5±8.1
Males	39 (97.5)
Ever-smokers	40 (100.0)
Current	8 (20.0)
Former	32 (80.0)
Chronic bronchitis	28 (70.0)
Exacerbations previous yr	0 (0–2)
Use of inhaled steroids	29 (72.5)
Post-bronchodilator	
FEV1 %	57.9±19.1
FVC %	89.4±23.1
FEV1/FVC %	50.4±10.2
***P*a,O2 mmHg**	76.8 (10.9)
***P*a,CO2 mmHg**	40.5 (3.5)

Data are presented as mean±SD, n (%), or median (interquartile range), unless otherwise stated. FEV1: forced expiratory volume in 1 s; FVC: forced vital capacity; P_{a,O_2}: arterial partial pressure of oxygen; P_{a,CO_2}: arterial partial pressure of carbon dioxide.

RESULTS

40 consecutive, clinically stable COPD outpatients fulfilled the inclusion criteria and were enrolled. Participating patients were mostly male (39 (97.5%)), had a mean age±SD of 66.5±8.1 yrs and showed moderate lung function impairment (post-bronchodilator FEV1 57.9% of predicted (SD 19.1%)) (table 1). In these patients, 79 baseline visits and consecutive follow-up examinations were performed after 8.0±3.2 months. Sputum was obtained with isotonic saline in 23 baseline and 34 follow-up visits, and was induced with hypertonic saline in the remaining examinations. PPMs were recovered from nearly three-quarters of these sputum samples, both at baseline and at

TABLE 2 Characteristics of sputum sample (n=79)

	Sputum		
	Baseline	Follow-up	p-value
PPMs	58 (73.4)	56 (70.9)	>0.25
Sputum neutrophilia#	53 (67.1)	40 (50.6)	>0.25
PPMs and neutrophilia#	45 (57.0)	35 (44.3)	0.15
Polymicrobial	10 (12.7)	9 (11.4)	>0.25
Microbial typing			
H. influenzae	28 (35.4)	25 (31.6)	>0.25
Load cfu·mL^{-1} × 10^3	100 (20–10000)	700 (10–4500)	>0.25
P. aeruginosa or enterobacteria	18 (22.8)	22 (27.8)	>0.25
Load cfu·mL^{-1} × 10^3	2 (1–10)	1 (1–6)	>0.25
H. parainfluenzae	16 (20.2)	16 (20.2)	>0.25
Load cfu·mL^{-1} × 10^3	100 (10–115)	55 (6–165)	>0.25
M. catarrhalis	4 (5.1)	2 (2.5)	>0.25
TNF-α pg·mL^{-1}	9 (5–29)	18 (4–41)	>0.25
IL-1β pg·mL^{-1}	664 (173–1619)	837 (179–2493)	>0.25
IL-6 pg·mL^{-1}	692 (182–2694)	494 (120–1834)	>0.25
IL-8 ng·mL^{-1}	31 (8–44)	40 (23–65)	0.02
IL-10 pg·mL^{-1}	6 (4–13)	10 (3–27)	0.16
IL-12 pg·mL^{-1}	7 (1–17)	7 (1–23)	>0.25

Data are present as n (%) and median (interquartile range). PPM: potentially pathogenic microorganisms; *H. influenzae*: *Haemophilus influenzae*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *H. parainfluenzae*: *Hemophilus parainfluenzae*; *M. catarrhalis*: *Moraxella catarrhalis*; cfu: colony-forming unit; TNF: tumour necrosis factor; IL: interleukin. #: Murray-Washington score ⩾3.

TABLE 3 Evolution during follow-up (n=79)

Months follow-up	8.0±3.2
⩾1 exacerbation during follow-up	21 (26.6)
Exacerbations in follow-up per yr	0 (0–1)
Change FEV1 post-bronchodilation mL·yr^{-1}	-72 (-270–120)
Former smokers#	-50 (-390–126)
Current smokers#	-76 (-240–90)
Sputum cultures baseline and follow-up	
PPMs in both	42 (53.2)
Same PPM strain in both	12 (15.2)
PPMs and neutrophilia¶ in both	16 (20.3)
Same PPM strain and neutrophilia¶ in both	12 (15.2)

Data are presented as mean±SD, n (%) or median (interquartile range). FEV1: forced expiratory volume in 1 s; PPM: potentially pathogenic microorganisms. #: difference statistically nonsignificant (p>0.25, Mann–Whitney U-test). ¶: Murray-Washington score ⩾3.

the follow-up visits, and in half of them this recovery was associated with a neutrophilic response. *H. influenzae* was the PPM recovered most often (in 28 and 25 cases at baseline and follow-up visits, respectively), and samples growing that microorganism also had the highest microbial loads. Low loads of *P. aeruginosa*, enterobacteria and *H. parainfluenzae*, however, were also often cultivated from the sputum samples (table 2).

59 samples showed bronchial colonisation at the follow-up examination, preceded by positive sputum cultures for PPMs in the baseline sputum sample in 42 (53.2%) cases. These successive positive cultures, however, were in most cases due to new PPMs acquired during follow-up (30 (38.0%) cases). Long-term persistence of the same strain was the cause in only 12 (15.2%) cases, a situation that was mainly attributable to colonisation by *P. aeruginosa* or enterobacteria (table 3). No statistically significant differences in the measured bronchial inflammation parameters were found between sputum samples with long-term persistent and acquired colonisation (table 4).

The identification of bronchial colonisation by *H. influenzae* in the follow-up sputum was associated with a bronchial inflammatory response identifiable through a higher prevalence of sputum neutrophilia in samples positive for this PPM (68.0 *versus* 42.6%; p=0.04, Chi-squared test), and higher concentrations of IL-1β (median 1,636 (IQR 597–7,736) *versus* 601 (153–1,320) pg·mL^{-1}; p=0.005, Mann–Whitney U-test) and IL-12 (14 (7–29) *versus* 2 (1–17) pg·mL^{-1}; p=0.01, Mann–Whitney U-test) (fig. 2; table 5). This inflammatory response was not observed in samples colonised by *P. aeruginosa*/enterobacteria or *H. parainfluenzae* (table 5). The recovered load of *H. influenzae* was also positively correlated with the sputum concentrations of IL-1β in patients with sputum cultures positive for this PPM (ρ=0.64, p=0.001, Spearman's correlation coefficient) (fig. 3). In samples colonised by *P. aeruginosa* or enterobacteria, a neutrophilic inflammatory response was also observed (p=0.05, Chi-squared test) (table 5). This neutrophilic inflammatory response, mainly found in patients colonised by *H. influenzae*, *P. aeruginosa* or enterobacteria (27 out of 40 neutrophilic samples, 67.5%), showed a statistically significant relationship with FEV1 declines over the median decline during follow-up in our sample of moderate COPD patients (OR 2.67, 95% CI 1.07–6.62; p=0.03). That relationship was not modified after adjustment for covariates and was not found for IL-1β or IL-12, nor were statistically significant relationships found between inhaled corticosteroid use or appearance of an exacerbation and follow-up inflammatory markers in sputum (data not shown).

DISCUSSION

In the present study, focusing on the effects of bronchial colonisation on inflammation and lung function in moderate COPD, we found that PPMs were recovered from bronchial secretions in nearly three-quarters of the patients. The recovery of *H. influenzae*, *P. aeruginosa* and enterobacteria from bronchial secretions was closely related to a neutrophilic response, suggesting that the cellular inflammatory effects of bronchial colonisation on moderate COPD depend mainly on the presence of these PPMs. *H. influenzae* colonisation was additionally associated with higher sputum concentrations of such inflammation markers as IL-1β and IL-12, a relationship that was shown to be load-mediated for IL-1β. Bronchial colonisation by *H. parainfluenzae*, however, was not associated with an inflammatory response, a finding suggesting that the effect of this microorganism on the bronchial mucosa must be considered as marginal. The repetition of the sputum cultures was recurrently positive for PPMs in more than half of the

TABLE 4 Microbial load and inflammatory markers in follow-up samples showing colonisation according to persistence of potentially pathogenic microorganisms

	PPM strain		
	Persistent	Acquired	p-value
Samples n	12	44	
H. influenzae	5 (41.7)	20 (42.5)	>0.25
Load[#] cfu·mL^{-1} × 10^3	3000 (50–10000)	550 (10–2750)	0.23
***P. aeruginosa* or enterobacteria**	9 (75.0)	13 (27.7)	0.006
Load[#] cfu·mL^{-1} × 10^3	1 (1–10)	1 (1–3)	>0.25
H. parainfluenzae	2 (16.7)	14 (29.8)	>0.25
Load[#] cfu·mL^{-1} × 10^3	10, 60[¶]	55 (6–165)	>0.25
Inflammatory markers			
Sputum neutrophilia[+]	8 (66.7)	27 (61.4)	>0.25
TNF-α pg·mL^{-1}	21 (9–56)	19 (3–48)	>0.25
IL-1β pg·mL^{-1}	1404 (662–2790)	1095 (188–5323)	>0.25
IL-6 pg·mL^{-1}	664 (156–2335)	557 (151–2532)	>0.25
IL-8 ng·mL^{-1}	42 (15–55)	41 (24–92)	>0.25
IL-10 pg·ml^{-1}	14 (6–31)	10 (3–29)	>0.25
IL-12 pg·mL^{-1}	16 (9–26)	7 (1–21)	0.08

Data are presented as n (%) or median (interquartile range), unless otherwise stated. PPM: potentially pathogenic microorganisms; *H. influenzae*: *Haemophilus influenzae*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *H. parainfluenzae*: *Hemophilus parainfluenzae*; cfu: colony-forming units; TNF: tumour necrosis factor; IL: interleukin. [#]: considering only samples with cultures positive for the potentially pathogenic microorganism; [¶]: absolute values for the only two individual observations; [+]: Murray–Washington score ⩾3. Comparisons were performed using a Chi-squared, Fisher's exact or Mann–Whitney U-test as required.

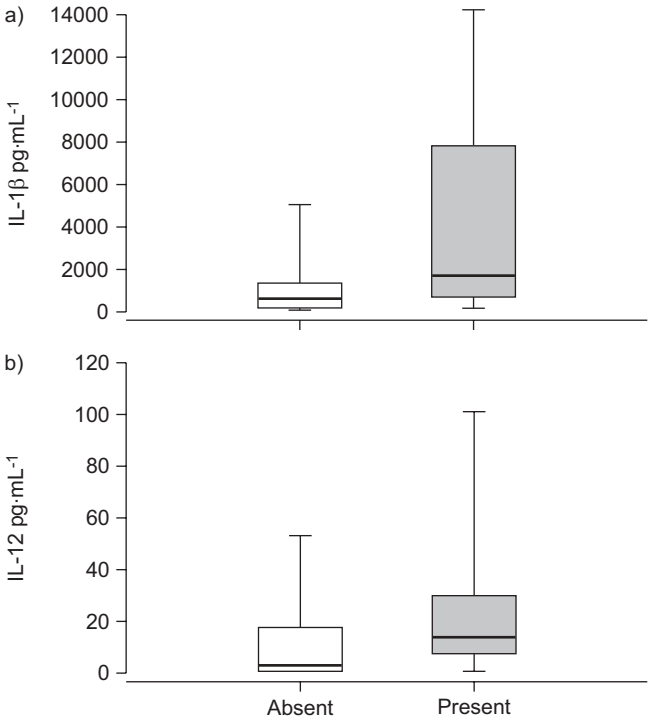

FIGURE 2. a) Interleukin (IL)-1β and b) IL-12 inflammatory markers in sputum according to the presence of bronchial colonisation by *Haemophilus influenzae*. Boxes represent median (horizontal dividing line) and interquartile range; whiskers represent SD.

patients, in most cases due to colonisation during follow-up by a newly acquired PPM; thus, long-term persistence of the same strain was observed in fewer than a fifth of the cases, all, however, with an associated neutrophilic response. The finding of sputum neutrophilia during stability was associated with a significant lung function decline in these moderate COPD patients, a relationship that was independent of current smoking.

Previous studies of bronchial colonisation in stable COPD patients, in which the protected specimen brush was used to collect microbiology samples, have demonstrated cultures positive for PPMs in one-third of the patients, with *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* the PPMs most often recovered [1]. When sputum has been used for the identification of bronchial colonisation in COPD, the cultures positive for PPMs have been found in one-fifth [5] to three-quarters [4] of the samples. In patients unable to spontaneously produce appropriate sputum samples during their stable periods, the use of sputum induction has facilitated the study of bronchial colonisation by allowing most stable COPD patients to be sampled [6, 27]. Study of the cytological characteristics of sputum in this context has shown cultures positive for PPMs with neutrophilia in half of the studied cases [3, 28], a rate similar to the prevalence found in the present study, in which *H. influenzae* was the most frequently isolated PPM.

Bronchial colonisation by *H. influenzae* was significantly related to sputum neutrophilia and higher sputum concentrations of the inflammatory mediators IL-1β and IL-12 in our sample of stable COPD patients with moderate lung function impairment. These observations suggest that colonisation by these PPMs is already able to induce an inflammatory response in the bronchial mucosa of such patients. This inflammatory response was seen to be species-specific because in our series, a similar effect was not found in COPD patients colonised by *H. parainfluenzae*, a PPM that occasionally causes COPD exacerbation and pneumonia [29–31], but has been shown to exhibit low mucosal adherence and a minimal potential to cause bronchial inflammation [32, 33]. Our choice of *H. influenzae* load as a main study variable was justified by its close relationship to the inflammatory response of the bronchial mucosa. Most PPMs recovered from participating COPD patients grew low microbial loads ($\leqslant 10^5$ cfu·mL^{-1}), with high microbial loads being found almost exclusively when *H. influenzae* was the colonising PPM in our study. High-load *H. influenzae* colonisation was significantly associated with neutrophilic sputum samples (Murray-Washington ⩾3) and higher levels of IL-1β in sputum. When dose–response relationships between PPM load and bronchial inflammatory mediators in sputum have been assessed in previous studies not focusing on *H. influenzae*, they have supported a causal role for bronchial colonisation in the pathogenesis of bronchial

TABLE 5 Inflammatory markers at follow-up according to sputum microbial typing (n=79)

	Colonisation by:								
	H. influenzae			*P. aeruginosa* or enterobacteria			*H. parainfluenzae*		
	Absent	Present	p-value#	Absent	Present	p-value#	Absent	Present	p-value#
Samples n	54	25		57	22		63	16	
Load cfu·mL^{-1} × 10^3		700 (10–4000)			1 (1–5)			5 (8–115)	
Sputum neutrophilia	23 (42.6)	17 (68.0)	0.04	25 (43.9)	15 (68.2)	0.05	30 (47.6)	10 (62.5)	>0.25
TNF-α pg·mL^{-1}	12 (4–33)	28 (4–56)	0.16	19 (4–43)	10 (5–46)	>0.25	19 (5–48)	14 (2–30)	>0.25
IL-1β pg·mL^{-1}	601 (153–1320)	1636 (597–7736)	0.005	601 (159–1809)	1126 (628–3630)	0.10	1037 (268–3956)	276 (113–830)	0.01
IL-6 pg·mL^{-1}	521 (67–1576)	463 (148–3539)	>0.25	468 (119–2326)	563 (186–1580)	>0.25	465 (126–1668)	647 (116–2421)	>0.25
IL-8 ng·mL^{-1}	38 (22–51)	41 (26–81)	0.24	37 (22–65)	41 (25–67)	>0.25	40 (25–65)	34 (16–61)	>0.25
IL-10 pg·mL^{-1}	9 (2–25)	14 (6–35)	0.09	12 (4–28)	6 (2–23)	>0.25	10 (4–26)	9 (2–29)	>0.25
IL-12 pg·mL^{-1}	2 (1–17)	14 (7–29)	0.01	7 (1–20)	9 (1–28)	>0.25	7 (1–26)	1 (1–10)	0.11

Data are presented median (interquartile range) or n (%), unless otherwise stated. *H. influenzae*: *Haemophilus influenzae*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *H. parainfluenzae*: *Hemophilus parainfluenzae*; cfu: colony-forming units; TNF: tumour necrosis factor; IL: interleukin. #: Comparisons were performed using a Chi-squared test, a Fisher's exact test or a Mann–Whitney U-test as required.

inflammation in COPD [4, 34, 35], and loads >10^5 cfu·mL^{-1} have usually been associated with neutrophilic inflammation [3]. Most series of patients with advanced disease, however, have shown positive correlations between microbial load and IL-8, myeloperoxidase, neutrophil elastase and leukotriene B_4 [3, 4, 28]. Together, all of these findings point to a significant impact of bronchial colonisation load on the mucosal inflammatory response, which, according to our results, may already be found, though with a different pattern, in patients with moderate disease when *H. influenzae* is the colonising bacteria. IL-1β has been related to bronchial colonisation in animal models [36], and our observation of high concentrations of this inflammatory mediator and IL-12, a cytokine related to the mucosal response against bacteria [37], in moderate COPD patients with *H. influenzae* colonisation supports the hypothesis of differences in the inflammatory response to bronchial colonisation according to the severity of lung function impairment. Our finding of a different pattern may have clinical implications and requires further assessment.

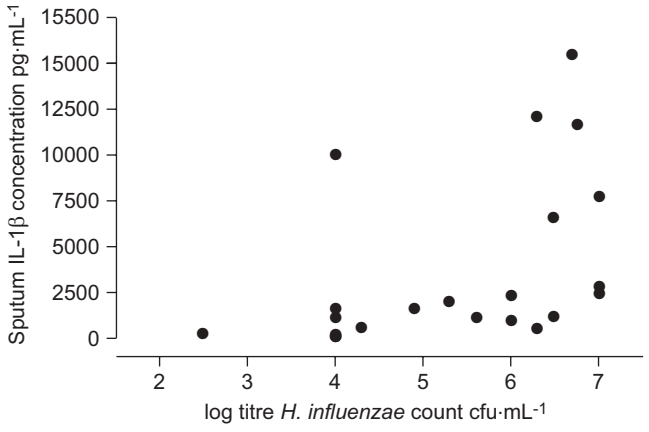

FIGURE 3. Scatterplot of total bacterial count (colony-forming units (cfu)·mL^{-1}) and sputum interleukin (IL)-1β (Spearman's correlation coefficient ρ=0.64, p=0.001) in patients colonised by *Haemophilus influenzae* at the follow-up examination (n=25). Bacterial count data have been logarithmically transformed.

We have found that samples showing bronchial colonisation were preceded by positive sputum cultures for PPMs in more than half the cases, and we have used molecular typing techniques to investigate whether recovered PPMs corresponded to newly acquired or long-term persistent strains. According to this analysis, bronchial colonisation was mostly attributable to new PPMs rather than to persistent ones. Persistent colonisation was observed in fewer than a fifth of the samples that were positive on follow-up and was most often attributable to long-term colonisation by *P. aeruginosa* or enterobacteria. Two previous studies reported that microbial persistence accounted for about a half of the cases of bronchial colonisation in stable COPD patients [3, 5], a prevalence higher than we have found. These studies, however, mostly investigated patients with severe COPD, and only one of them used molecular typing techniques to rule out colonisation by a new strain of the same PPM [5]. Our observations suggest that, in spite of a high prevalence of bronchial colonisation in moderate COPD patients, long-term microbial persistence is less often the cause, probably due to a higher turnover of colonising strains in less advanced disease.

Our findings in moderate COPD patients colonised by *H. influenzae*, *P. aeruginosa* and enterobacteria confirm the close association between colonisation by these PPMs and neutrophilia reported in other studies [34]. This association supports

an early relationship between colonisation and the appearance of cellular inflammatory mediators in bronchial secretions, years before the disease causes severe impairment and at a stage when these relationships could be more easily identifiable [38, 39]. The importance of the neutrophilic inflammatory response associated with bronchial colonisation is emphasised by our finding of a statistically significant relationship between the appearance of this inflammatory response and lung function impairment during follow-up. A similar functional impairment associated with bronchial colonisation has been documented by WILKINSON *et al.* [3], but was not found by HILL *et al.* [4]. In our study, the decline in FEV1 in COPD patients with sputum neutrophilia in some cases approached the magnitude previously seen in exacerbated COPD patients with more severe disease [40]. This finding supports the hypothesis that bronchial colonisation may have a subclinical impact in moderate COPD patients, who may be less prone to report their daily symptoms as an exacerbation [41]. Such patients may have functional impairment equivalent to the FEV1 decline that is associated with exacerbation in patients with advanced disease, who suffer easily from dyspnoea with even slight impairments in their lung function.

We have not performed genomic analysis of the sputum samples that do not grow PPMs, and we cannot rule out underdiagnosis of microbial persistence due to bronchial colonisation at loads below the detection limit of the sputum culture. This situation must be considered as unusual, however, because when this approach has been used undiagnosed, bronchial colonisation has only been identified in one-tenth of the culture-negative sputum samples [42]. The lack of virological data is also a limitation of our study, but respiratory viruses are seldom recovered from bronchial secretions in stable COPD patients, who only give positive samples in one-tenth of the cases [43, 44]. The lowest prevalence of viral recovery has been found in infrequent exacerbators [44], such as those in our study.

In summary, we found that the bronchial tree of clinically stable outpatients with moderate COPD and without previous admissions due to respiratory diseases is often colonised by PPMs, but high sputum loads are only reached by *H. influenzae*. The presence of this PPM is associated with an inflammatory response of the bronchial mucosa, characterised by neutrophilic inflammation and high levels of IL-1β and IL-12, findings that are not characteristic of patients colonised by *H. parainfluenzae*. Colonising *P. aeruginosa* and enterobacteria are more often persistent and also associated with sputum neutrophilia. These observations suggest that the effect of bronchial colonisation on COPD patients is mainly mediated by *H. influenzae*, *P. aeruginosa* and enterobacteria, PPMs that are able to cause an identifiable inflammatory response in moderately ill COPD patients.

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STATEMENT OF INTEREST

None declared.

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