High Blood Eosinophil Count at Stable State is Not Associated with Airway Microbiota Distinct Profile in COPD

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ORIGINAL RESEARCH

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Title: High blood eosinophil count at stable state is not associated with airway microbiota distinct profile in COPD

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Abstract

Purpose: The heterogeneity of clinical features in COPD at stable state has been associated with airway microbiota. Blood eosinophil count (BEC) represents a biomarker for a pejorative evolution of COPD, including exacerbations and accelerated FEV_1 decline. We aimed to analyse the associations between BEC and airway microbiota in COPD at stable state.

Patients and methods: Adult COPD patients at stable state (RINNOPARI cohort) were included and characterised for clinical, functional, biological and morphological features. BEC at inclusion defined 2 groups of patients with low BEC <300/mm^3 and high BEC ≥300/mm^3. Sputa were collected and an extended microbiological culture was performed for the identification of viable airway microbiota.

Results: Fifty-nine subjects were included. When compared with the low BEC (n=40, 67.8%), the high BEC group (n=19, 32.2%) had more frequent exacerbations (p<0.001) and more pronounced cough and sputum (p<0.05). The global composition, the number of bacteria per sample and the α-diversity of the microbiota did not differ between groups, as well as the predominant phyla (Firmicutes), or the gender repartition.

Conclusion: In our study, high BEC in COPD at stable state was associated with a clinical phenotype including frequent exacerbation, but no distinct profile of viable airway microbiota compared with low BEC.

Key words: COPD, Eosinophil, sputum, microbiota

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Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by chronic respiratory symptoms including dyspnea, cough, sputum production, and exacerbations, due to abnormalities of the airways and/or alveoli that cause persistent, often progressive airflow limitation (1). COPD is a heterogeneous disease regarding clinical features, with high morbidity and mortality. The pathogenesis of COPD is still not completely elucidated, including structural and inflammatory changes (1). Blood eosinophil count (BEC) has been recently identified as a biomarker of interest in stable COPD. Greater BEC levels have been associated with greater FEV_1 decline in the absence of inhaled corticosteroid (ICS) treatment, and with the risk of exacerbation (1, 2). It is currently recommended to use BEC, not as a standalone biomarker but together with exacerbation history, to identify patients with the greatest likelihood of ICS treatment benefit (1, 3).

Airway microbiota at stable state might participate in the heterogeneity of COPD clinical presentation. Previous studies performed in a stable state identified associations between the sputum microbiome and COPD phenotypes (4-6). Associations with COPD inflammatory phenotypes including eosinophils have been recently investigated, suggesting that different profiles of bacteria phyla might be associated with different profiles of inflammation: low sputum eosinophil count in the presence of potential pathogenic microorganism (PPM) (7, 8), more diverse respiratory microbiome in subjects with BEC ≥2% (9). However, the relationships between BEC and airway microbiota and its clinical implications remain to be determined.

The most frequently used techniques for airway microbiota analyses (PCR amplification, sequencing of the bacterial 16S ribosomal RNA gene) do not involve bacterial culture. We previously described an extended conventional culture-based approach, allowing the identification of bacteria restricted to viable strains (6, 10). In this study, we applied this culture technique and compared viable airway microbiota in COPD patients at a stable state with high and low BEC.

Methods

Patients

Patients with mild to severe COPD were included prospectively in the RINNOPARI cohort (Recherche et INNOvation en PAthologie Respiratoire Inflammatoire; University Hospital of Reims, France; NCT02924818) as described previously (6) The regional ethics committee approved the study (Comité de Protection des Personnes—Dijon EST I, no. 2016-A00242-49). All patients provided written consent. Exclusion criteria were other pulmonary diseases, including asthma, bronchiectasis, cystic fibrosis, bronchopulmonary allergic aspergillosis, (CF), or pulmonary fibrosis. Stable state was defined as 4 weeks or more from the last exacerbation (defined as an acute worsening of respiratory symptoms resulting in additional therapy (6, 11). COPD was defined by postbronchodilator $FEV_1/FVC < 70\%$. The severity of COPD was determined by spirometric classification (GOLD 1: $FEV_1 \geq 80\%$; GOLD 2: $50\% \leq FEV_1 < 80\%$; GOLD 3: $30\% \leq FEV_1 < 50\%$; GOLD 4: $FEV_1 < 30\%$), and ABE classification depending on exacerbations, dyspnea and CAT score (1). Emphysema was visually assessed and quantified from CT scan images as previously described (12, 13). BEC was performed at inclusion, defining 2 groups of patients with low BEC $<300/mm^3$ and high BEC $\geq 300/mm^3$, using the cut-off proposed by GOLD 2023 (1).

Sputum analysis

Sputa were collected and an extended microbiological culture was performed, as previously described (6). After liquefaction by N-acetylcysteine, serial dilutions (from 1/1,000 to 1/100,000) of the sputum were performed and cultured at 37°C for 48 h (aerobic cultures), or 5% CO_2 for 5 days (anaerobic cultures) on several agar media including Columbia blood, chocolate, Schaedler, and *Pseudomonas*-selective cetrimide (Thermo Fisher Scientific, USA) We next quantified all morphologically distinct colonies as colony-forming unit (CFU) per milliliter. Colonies were then identified using MALDI-TOF mass spectrometry (MALDI

Biotyper®, Bruker Daltonics, Germany). We estimated the viable airway microbiota α-diversity using the Shannon index.

Statistical Analysis

The descriptive data are expressed as numbers (percentages), median [25th-75th quartiles], or mean values ± standard deviation, when appropriate. Qualitative variables were compared by the chi-square test or Fisher exact test. Quantitative variables were compared using the t-test or Mann–Whitney test. A p-value < 0.05 was considered significant. The dissimilarities in bacterial communities between low BEC <300/mm3 and high BEC ≥300/mm3 groups were visualised in a low-dimensional Euclidean space, using unsupervised principal component analysis (PCA), that was plotted along the first two principal components (the two explaining most of the variance).

Results

Patients

Fifty-nine subjects were included in the study, 40 in the low BEC group (BEC<300/mm^3, 67.8%) and 19 in the high BEC group (BEC ≥ 300/mm^3, 32.2%). Subjects' characteristics are detailed in Table 1 and Suppl Table 1. Briefly, they were predominantly men (57.6%), mean age 61 ± 9 years. COPD was severe to very severe in 57.6%, 66.1% had one or more exacerbations in the last year, with a median of 2 exacerbations. Two patients received long term oral corticosteroids at inclusion, including one in low BEC group, and one in high BEC group, with no significant differences between groups (Table 1). When compared with the low BEC group, the high BEC group was characterised by a higher number of exacerbations per patient (median 2 [0-4] vs 1 [0-3], p<0.001), more pronounced symptoms of cough and sputum (p<0.05, CASA-Q, Table 1) and a trend for lower smoking exposure (p=0.06) and more frequent antibiotic course (p=0.06). We did not find any differences in terms of inhaled treatment, lung function, or emphysema score.

Microbiology

The extended culture method was applied to 59 sputa (1 sample per patient) to determine the viable airway microbiota. We identified 386 bacteria, from 71 different species, representing a mean of 6.6 bacteria per sample (Table S2). We compared the viable microbiota of the low and high BEC groups by PCA analysis and we found no difference in the global composition of the microbiota of the 2 groups (Figure 1A). The number of bacteria per sample and the α-diversity of the microbiota did not differ between low and high BEC groups (Figure 1B and C). The repartition of the bacterial phyla was similar in the 2 groups, with a predominance of Firmicutes (Figure 1D). We observed the same repartition of different genera: *Streptococcus, Rothia, Veillonella, Neisseria,* and *Actinomyces* were predominant and represented more than 65% of the bacteria identified (Figure 1E). The prevalence of the different species in the 2 groups was analysed and no difference was found between the low and high BEC groups (Figure 1F). The most common bacteria in both groups were *Streptococcus oralis/mitis/pneumoniae*, identified in more than 90% of samples, followed by *Veillonella parvula/dispar/atypica* found in more than 50% of samples. Bacterial quantifications of the viable microbiota ranged from 10^2 CFU/mL to 10^9 CFU/mL, with a median of 10^6 CFU/mL and no difference between the 2 groups (Table S2).

In this cohort of COPD patients at stable state, some PPM were detected*: Staphylococcus aureus* (n = 8, 13,6%), *Haemophilus influenzae* (n = 6, 10.2%), *Moraxella catarrhalis* (n = 5, 8.5%) and *Pseudomonas aeruginosa* (n = 3, 5.1%), with the same prevalence in the 2 groups (n=16; 40.0% in the low BEC group *vs* n= 7, 36.8% in the high BEC group) (Table S2).

Discussion

In this cross-sectional analysis focusing on COPD patients at stable state and using an original extended conventional culture-based approach that allows the identification of

bacteria restricted to viable strains, we identified that high BEC is not associated with a distinct profile of viable airway microbiota, despite a BEC-associated clinical phenotype.

In our study, the high BEC group was characterised by a clinical phenotype including more frequent exacerbations in the last year, a trend for a more frequent antibiotic course, and more pronounced cough and sputum symptoms (CASA-Q). Elevated BEC at a stable state has been previously identified as a biomarker for exacerbation risk in COPD, with an incidence rate ratio of exacerbation of 1.32 for BEC ≥300/mm^3 in the COPDgene study (2). An association between sputum inflammatory cells and symptoms at stable state has been shown, with neutrophilic inflammation being associated with cough, and eosinophilic inflammation with dyspnea (14, 15). A recent post-hoc analysis suggested that COPD patients with both high eosinophil levels in sputum (≥3%) and chronic bronchitis might present a distinct profile of gene expression, characterised by the overexpression of T2- and phosphodiesterase-4-inhibitors-related genes (16). The high BEC group in our study was further characterised by a trend for lower smoking exposure, but a similarly altered lung function. This might indirectly reflect the previously observed accelerated lung function decline in COPD subjects with high eosinophil counts (17).

We did not identify a distinct profile of viable airway microbiota in the high BEC group when compared with the low BEC group. Firmicutes and *Streptococcus* were the predominant bacteria phylum and species respectively, in line with previous analyses performed in COPD at a stable state (5, 18). The previously reported decrease in Proteobacteria abundance and increase in Firmicutes phyla in subjects with high BEC (19), or the more diverse microbiome in subjects with BEC ≥2% (9) were not found in our study. It must be pointed out that these previous studies used gene sequencing techniques (16S rRNA), which are not able to discriminate between viable and non-viable strains. Previous studies described the presence of PPM in subjects with low sputum eosinophil count (7, 8). However, these studies used qPCR detection restricted to 3 species (*H. influenzae*, *M. catarrhalis,* and *S. pneumoniae*),

178 therefore also including both viable and non-viable strains. Our study did not confirm those
179 results.

180 Our study suffers from several limits. Although monocentric, the patient's recruitment was
181 prospective and all benefited from an in-depth phenotypic characterisation. Exacerbation
182 frequency was estimated retrospectively in the last year before inclusion. We performed only
183 a one-point BEC assessment at inclusion, while BEC is known to vary over time in COPD
184 (20). However, the clinical phenotype of the high BEC group including more frequent
185 exacerbation matches with previous studies (2). Inhaled treatment was heterogeneous with
186 32% of the patients using ICS. ICS treatment can alter the microbiome in the small airways
187 of patients with COPD and might have an impact on our results (21). However, inhaled
188 treatment strategies including ICS in our study did not significantly differ between high and
189 low BEC groups. Finally, our extended conventional culture-based approach may have lower
190 sensitivity than a metagenomic approach for a more in-depth characterisation of the lung
191 microbiota.

192

193 **Conclusion**

194 In our study using an original extended conventional culture-based approach, high BEC in
195 COPD at a stable state was associated with a clinical phenotype including frequent
196 exacerbation and more cough and sputum symptoms, but no distinct profile of viable airway
197 microbiota compared with low BEC.

198

Declarations

Ethics approval and informed consent

The study was approved by the regional ethics committee (Comité de Protection des Personnes—Dijon EST I, no. 2016-A00242-49). Informed consent was obtained from all the patients.

Consent for publication

Not applicable

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Competing interests

J.M. Perotin reports lecture honoraria from AstraZeneca, and support for attending meetings from AstraZeneca and Chiesi; outside the submitted work. C. Launois reports support for attending meeting from Chiesi; outside the submitted work. V. Dormoy reports lecture honoraria from Chiesi and AstraZeneca; outside the submitted work. G. Deslée reports lecture honoraria from Chiesi, AstraZeneca and GlaxoSmithKline; outside the submitted work. There are no further conflicting interests to disclose.

Authors' contributions

Study concept: J.M. Perotin, A. Muggeo, T. Guillard and G. Deslee; study design: J.M. Perotin and A. Muggeo; acquisition data: J.M. Perotin, A. Muggeo, Q. Lecomte, S. Dury, C. Launois, J. Ancel and V. Dormoy; analysis and data interpretation: J.M. Perotin, A. Muggeo, Q. Lecomte, A. Brisebarre, S. Dury, C. Launois, J. Ancel, V. Dormoy, T. Guillard and G. Deslee; revision of manuscript: J.M. Perotin, A. Muggeo, Q. Lecomte, A. Brisebarre, S. Dury, C. Launois, J. Ancel, V. Dormoy, T. Guillard and G. Deslee; manuscript writing: J.M. Perotin, A. Muggeo, T. Guillard and G. Deslee.

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288 **Tables**

289 Table 1. Patients' characteristics

	Total	Low BEC	High BEC	p-value
n	59	40	19	
Age, yrs	61.0 ± 9.3	61.3 ± 9.6	60.4 ± 8.9	0.365
Men	34 (57.6)	22 (55.0)	12 (63.2)	0.380
BMI, kg/m²	25.8 ± 5.7	25.7 ± 6.1	25.9 ± 4.8	0.440
Current smoker	21 (35.6)	15 (37.5)	6 (31.6)	0.443
Smoking history, pack-year	43.4 ± 19.5	46.1 ± 18.6	37.6 ± 20.5	0.059
Inhaled treatment strategy				
No inhaled treatment	11 (18.6)	5 (12.5)	6 (31.6)	
LABA or LAMA	5 (8.5)	4 (10.0)	1 (5.3)	
LABA + LAMA	24 (40.7)	18 (45.0)	6 (31.6)	0.204
ICS + LABA or ICS +LAMA	5 (8.5)	2 (5.0)	3 (15.8)	
ICS + LAMA + LABA	14 (23.7)	11 (27.5)	3 (15.8)	
Long-term oral corticosteroids	2 (3.4)	1 (2.5)	1 (5.3)	0.643
Long-term oxygen	10 (17.0)	7 (17.5)	3 (15.8)	0.561
Exacerbation in the previous year, n	39 (66.1)	27 (67.5)	12 (63.2)	0.481
Number per patient	1 [0-3]	1 [0-3]	2 [0-4]	0.001
Antibiotics (6 Mo, nb/patient)	1 [0-1]	1 [0-1]	1 [0-3]	0.06
Dyspnea mMRC ≥ 2	44 (77.2)	31 (81.6)	13 (68.4)	0.323
CAT score	18.4 ± 7.4	17.7 ± 7.4	19.8 ± 7.7	0.165
CASA-Q scores				
Symptoms: cough	65.5 ± 24.0	70.2 ± 23.2	56.1 ± 23.2	0.018
Symptoms: sputum	64.9 ± 25.9	69.5 ± 26.8	55.7 ± 21.9	0.029
Impact: cough	72.4 ± 24.4	74.6 ± 26.4	67.9 ± 19.5	0.168
Impact: sputum	75.2 ± 22.3	78.2 ± 22.8	69.3 ± 20.6	0.079
Blood eosinophils, G/L	0.2 [0.1-0.3]	0.1 [0.1-0.2]	0.3 [0.3-0.4]	< 0.001
Total IgE, IU/mL	76 [20-256]	54 [18-339]	102 [45-418]	0.267
Positive fungal sputum analysis	20 (34.5)	16 (40.0)	4 (21.0)	0.239

290 Values are n (%), mean ± SD and median [25^{th}-75^{th}]. BMI: body mass index; LABA: long-
291 acting beta agonist; LAMA: long-acting muscarinic antagonist; ICS: inhaled corticosteroid;
292 CAT: COPD assessment test

293

294 **Figure**

295 Figure 1: Bacterial analysis of airway microbiota in low and high BEC groups. A: Principal
296 component analysis (PCA) of the airway microbiota. The axes are the first eigenvalues, the
297 ones explaining the most variance of the dataset. Individual patients are represented in blue
298 triangles (high BEC group, n=19) and green dots (low BEC group, n=40). The larges blue
299 triangle and red dot at the center of the ellipses represent the 95% confidence interval. B:
300 Number of species per sample. C: Alpha diversity of viable microbiota (Shannon index). D:
301 Phyla distribution. E: Genus distribution. F: Bacteria prevalence (note: bacteria with less than
302 5% frequency are not listed)