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1 ORIGINAL RESEARCH

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3 Perotin et al

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6 **Title: High blood eosinophil count at stable state is not associated with airway**

7 **microbiota distinct profile in COPD**

8

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21

22 **Abstract**

23 **Purpose:** The heterogeneity of clinical features in COPD at stable state has been associated
24 with airway microbiota. Blood eosinophil count (BEC) represents a biomarker for a pejorative
25 evolution of COPD, including exacerbations and accelerated FEV₁ decline. We aimed to
26 analyse the associations between BEC and airway microbiota in COPD at stable state.

27 **Patients and methods:** Adult COPD patients at stable state (RINNOPARI cohort) were
28 included and characterised for clinical, functional, biological and morphological features. BEC
29 at inclusion defined 2 groups of patients with low BEC <300/mm³ and high BEC ≥300/mm³.
30 Sputa were collected and an extended microbiological culture was performed for the
31 identification of viable airway microbiota.

32 **Results:** Fifty-nine subjects were included. When compared with the low BEC (n=40,
33 67.8%), the high BEC group (n=19, 32.2%) had more frequent exacerbations (p<0.001) and
34 more pronounced cough and sputum (p<0.05). The global composition, the number of
35 bacteria per sample and the α-diversity of the microbiota did not differ between groups, as
36 well as the predominant phyla (Firmicutes), or the gender repartition.

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

40

41 **Key words:** COPD, Eosinophil, sputum, microbiota

42

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47 **Introduction**

48 Chronic obstructive pulmonary disease (COPD) is characterized by chronic respiratory
49 symptoms including dyspnea, cough, sputum production, and exacerbations, due to
50 abnormalities of the airways and/or alveoli that cause persistent, often progressive airflow
51 limitation (1). COPD is a heterogeneous disease regarding clinical features, with high
52 morbidity and mortality. The pathogenesis of COPD is still not completely elucidated,
53 including structural and inflammatory changes (1). Blood eosinophil count (BEC) has been
54 recently identified as a biomarker of interest in stable COPD. Greater BEC levels have been
55 associated with greater FEV₁ decline in the absence of inhaled corticosteroid (ICS)
56 treatment, and with the risk of exacerbation (1, 2). It is currently recommended to use BEC,
57 not as a standalone biomarker but together with exacerbation history, to identify patients with
58 the greatest likelihood of ICS treatment benefit (1, 3).

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

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

74

75 **Methods**

76 Patients

77 Patients with mild to severe COPD were included prospectively in the RINNOPARI cohort
78 (Recherche et INNOVation en PAtHologie Respiratoire Inflammatoire; University Hospital of
79 Reims, France; NCT02924818) as described previously (6) The regional ethics committee
80 approved the study (Comité de Protection des Personnes—Dijon EST I, no. 2016-A00242-
81 49). All patients provided written consent. Exclusion criteria were other pulmonary diseases,
82 including asthma, bronchiectasis, cystic fibrosis, bronchopulmonary allergic aspergillosis,
83 (CF), or pulmonary fibrosis. Stable state was defined as 4 weeks or more from the last
84 exacerbation (defined as an acute worsening of respiratory symptoms resulting in additional
85 therapy (6, 11). COPD was defined by postbronchodilator $FEV_1/FVC < 70\%$. The severity of
86 COPD was determined by spirometric classification (GOLD 1: $FEV_1 \geq 80\%$; GOLD 2: $50\% \leq$
87 $FEV_1 < 80\%$; GOLD 3: $30\% \leq FEV_1 < 50\%$; GOLD 4: $FEV_1 < 30\%$), and ABE classification
88 depending on exacerbations, dyspnea and CAT score (1). Emphysema was visually
89 assessed and quantified from CT scan images as previously described (12, 13). BEC was
90 performed at inclusion, defining 2 groups of patients with low BEC $< 300/mm^3$ and high BEC
91 $\geq 300/mm^3$, using the cut-off proposed by GOLD 2023 (1).

92 Sputum analysis

93 Sputa were collected and an extended microbiological culture was performed, as previously
94 described (6). After liquefaction by N-acetylcysteine, serial dilutions (from 1/1,000 to
95 1/100,000) of the sputum were performed and cultured at 37°C for 48 h (aerobic cultures), or
96 5% CO₂ for 5 days (anaerobic cultures) on several agar media including Columbia blood,
97 chocolate, Schaedler, and *Pseudomonas*-selective ceftrimide (Thermo Fisher Scientific, USA)
98 We next quantified all morphologically distinct colonies as colony-forming unit (CFU) per
99 milliliter. Colonies were then identified using MALDI-TOF mass spectrometry (MALDI

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

102 Statistical Analysis

103 The descriptive data are expressed as numbers (percentages), median [25th-75th quartiles],
104 or mean values \pm standard deviation, when appropriate. Qualitative variables were compared
105 by the chi-square test or Fisher exact test. Quantitative variables were compared using the t-
106 test or Mann–Whitney test. A p-value < 0.05 was considered significant. The dissimilarities in
107 bacterial communities between low BEC $<300/\text{mm}^3$ and high BEC $\geq 300/\text{mm}^3$ groups were
108 visualised in a low-dimensional Euclidean space, using unsupervised principal component
109 analysis (PCA), that was plotted along the first two principal components (the two explaining
110 most of the variance).

111

112 Results

113 Patients

114 Fifty-nine subjects were included in the study, 40 in the low BEC group (BEC $<300/\text{mm}^3$,
115 67.8%) and 19 in the high BEC group (BEC $\geq 300/\text{mm}^3$, 32.2%). Subjects' characteristics are
116 detailed in Table 1 and Suppl Table 1. Briefly, they were predominantly men (57.6%), mean
117 age 61 ± 9 years. COPD was severe to very severe in 57.6%, 66.1% had one or more
118 exacerbations in the last year, with a median of 2 exacerbations. Two patients received long
119 term oral corticosteroids at inclusion, including one in low BEC group, and one in high BEC
120 group, with no significant differences between groups (Table 1). When compared with the
121 low BEC group, the high BEC group was characterised by a higher number of exacerbations
122 per patient (median 2 [0-4] vs 1 [0-3], $p<0.001$), more pronounced symptoms of cough and
123 sputum ($p<0.05$, CASA-Q, Table 1) and a trend for lower smoking exposure ($p=0.06$) and
124 more frequent antibiotic course ($p=0.06$). We did not find any differences in terms of inhaled
125 treatment, lung function, or emphysema score.

126 Microbiology

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

143 In this cohort of COPD patients at stable state, some PPM were detected: *Staphylococcus*
144 *aureus* (n = 8, 13,6%), *Haemophilus influenzae* (n = 6, 10.2%), *Moraxella catarrhalis* (n = 5,
145 8.5%) and *Pseudomonas aeruginosa* (n = 3, 5.1%), with the same prevalence in the 2
146 groups (n=16; 40.0% in the low BEC group vs n= 7, 36.8% in the high BEC group) (Table
147 S2).

148

149 **Discussion**

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

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

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

168 We did not identify a distinct profile of viable airway microbiota in the high BEC group when
169 compared with the low BEC group. Firmicutes and *Streptococcus* were the predominant
170 bacteria phylum and species respectively, in line with previous analyses performed in COPD
171 at a stable state (5, 18). The previously reported decrease in Proteobacteria abundance and
172 increase in Firmicutes phyla in subjects with high BEC (19), or the more diverse microbiome
173 in subjects with BEC $\geq 2\%$ (9) were not found in our study. It must be pointed out that these
174 previous studies used gene sequencing techniques (16S rRNA), which are not able to
175 discriminate between viable and non-viable strains. Previous studies described the presence
176 of PPM in subjects with low sputum eosinophil count (7, 8). However, these studies used
177 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

199 **Declarations**

200 **Ethics approval and informed consent**

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

204 **Consent for publication**

205 Not applicable

206 **Data availability**

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

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211 **Competing interests**

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

218 **Authors' contributions**

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

226

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286

287

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/m ²	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 [25th-75th]. 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 largest 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)