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Robust airway microbiome signatures in acute respiratory failure and hospital-acquired pneumonia

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1 **TITLE**

2 **Robust airway microbiome signatures in acute respiratory failure and hospital-acquired**
3 **pneumonia**

4 **Running head:** Microbiome Signatures in Acute Respiratory Failure

5

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11

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38

39 **ABSTRACT**

40 Respiratory microbial dysbiosis is associated with acute respiratory distress syndrome (ARDS) and
41 hospital-acquired pneumonia (HAP) in critically ill patients. However, we lack reproducible respiratory
42 microbiome signatures that can increase our understanding of these conditions and potential treatments.
43 Here we analyzed 16S rRNA sequencing data from 2,177 respiratory samples collected in 1,029
44 critically ill patients (21.7% with ARDS and 26.3% with HAP) and 327 healthy controls from 17
45 published studies. After data harmonization and pooling of individual patient data, we identified
46 microbiota signatures associated with ARDS, HAP, and prolonged mechanical ventilation (MV). HAP
47 and prolonged MV microbiota signatures were characterized by depletion of a core group of microbes
48 typical of healthy respiratory samples, and ARDS signature was distinguished by enrichment of
49 potentially pathogenic respiratory microbes, including *Pseudomonas* and *Staphylococcus*. Using
50 machine learning models, we identified clinically informative, three- and four-factor signatures that
51 associated with ARDS, HAP, and prolonged MV with fair accuracy (AUC 0.751, 0.72, and 0.727,
52 respectively). We validated the signatures in an independent prospective cohort of 136 mechanically
53 ventilated patients and found that patients with ARDS, HAP, or prolonged MV microbiome signatures
54 had longer times to successful extubation than patients lacking these signatures (Hazard Ratio 1.56
55 (95%CI 1.07-2.27), 1.51 (95%CI 1.02-2.23) and 1.50 (95%CI 1.03-2.18), respectively). Thus we
56 defined and validated robust respiratory microbiome signatures associated with ARDS and HAP that
57 may help identify promising targets for microbiome therapeutic modulation in critically ill patients.

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59 **Keywords:** microbiome, acute respiratory distress syndrome, hospital-acquired pneumonia, dysbiosis,
60 critical illness.

61 INTRODUCTION

62 Acute respiratory distress syndrome (ARDS) and hospital-acquired pneumonia (HAP) are the most
63 frequent respiratory complications and important drivers of prolonged mechanical ventilation (MV) and
64 death in critically ill patients ^{1,2}. Despite advances in defining optimal MV settings and antimicrobial
65 therapies, adjunctive treatment options remain limited for ARDS and HAP ³⁻⁵. A better understanding
66 of the underlying pathophysiology of these conditions is needed to enable the development of innovative
67 therapeutics. There is a clinical overlap between ARDS and HAP since secondary pulmonary infections
68 are frequent complications of ARDS patients, and severe HAP can also evolve towards ARDS ⁶. The
69 comparative investigation of ARDS and HAP can increase our understanding of the cause of these
70 medical conditions.

71 Culture-independent, high-throughput sequencing of nucleic acids extracted from respiratory secretion
72 samples has shown that the lungs harbor highly diverse microbial communities, collectively known as
73 the lung microbiome, despite traditional dogmas of presumed lung sterility. ^{7,8} A group of five bacteria
74 found in most healthy individuals, composed of five genera *Prevotella*, *Streptococcus*, *Veillonella*,
75 *Fusobacterium*, and *Haemophilus*, has been proposed as a pulmonary core microbiota essential for lung
76 homeostasis ⁹⁻¹². Microbiome investigations in intensive care unit (ICU) patients have provided
77 supportive evidence for respiratory microbial dysbiosis in patients with ARDS ¹³⁻¹⁷ and HAP ¹⁸⁻²⁰.
78 Reproducible microbiome signatures have not been developed because most data were generated in
79 single-center studies with limited statistical power. Moreover, well-recognized limitations for data
80 synthesis across microbiome studies, such as variability of sample collection methods, DNA extraction,
81 and sequencing protocols, have hampered the generalizability of results and clinical translation at the
82 bedside.

83 The features of HAP and ARDS respiratory microbiome dysbiosis have not been thoroughly compared,
84 and this knowledge gap has limited the development of microbiome-targeted interventions for the
85 prevention and treatment of these conditions ²¹. We hypothesized that the comparative investigation of
86 the respiratory dysbiosis associated with ARDS and HAP would enable the identification of specific
87 signatures, thus improving understanding of the links and differences between these two conditions and
88 paving the way to developing common and specific innovative microbiome-targeted approaches. Thus,
89 we aimed to define robust respiratory microbiome signatures relevant to the pathophysiology of ARDS
90 and HAP using publicly available data and validation in an independent cohort.

91

92 Results

93 *Summary of available data*

94 We included 17 studies with 16S gene-based microbiome that had studied the respiratory microbiome
95 of patients hospitalized in ICUs or from healthy controls, from multiple countries (United States,
96 Switzerland, Germany, and China). Study characteristics are described in **Tables S1 to S3**. All studies
97 reporting ARDS outcomes used the diagnosis criteria meeting the Berlin definition for mild to severe

98 ARDS²² (n=9). All the studies reporting HAP occurrence used the clinical and radiological HAP criteria
99 in compliance with international guidelines^{23,24} (n=7), and 3 (43%) also included microbiological
100 criteria. We gathered individual data from 2177 respiratory samples, including 310 bronchoalveolar
101 lavages (BAL), 1083 endotracheal aspirates (ETA), and 784 oropharyngeal swabs (OS) obtained from
102 1029 critically ill patients. The median time of the sample collection was day 1 (IQR 1-5) after ICU
103 admission. Acute respiratory distress syndrome and HAP were diagnosed in 223 (21.6%) and 271
104 (26.3%) patients. One hundred seventy-three (11.2%) samples were collected in patients with both HAP
105 and ARDS criteria (**Table S4**). Data from 327 samples collected in healthy individuals were obtained
106 from four studies^{8,25-27}.

107 **Characterization of respiratory microbiota in critically ill patients**

108 Here, we assessed if samples collected in critically ill patients (n=2177) differed from those collected in
109 healthy individuals (n=327). We initially observed that the respiratory microbiota of all samples (BAL,
110 ETA, and OS) collected during ICU hospitalization were distinct from those obtained from healthy
111 individuals using Principal Coordinates Analysis (PCoA) (p-value = 0.001, r² = 0.016; **Figure 1A**). The
112 differences between ICU patients and healthy controls were also found when BAL, ETA and OS
113 samples were analyzed separately (**Figure S1A**). We then studied the alpha diversity of the respiratory
114 microbiome. This measure summarizes species abundance distribution in each sample into a single
115 number and depends on species richness (number of species in a sample) and relative abundance
116 (evenness). Alpha diversity was lower in critically ill patients than healthy controls (**Figure 1B** for all
117 samples, **and S1B** in BAL). Among ICU patients, alpha diversity in the samples collected following
118 five days of hospitalization was lower than those collected before Day 4 (**Figure S1C**).

119 In the 2117 samples from critically ill patients, we found that five of the six most prevalent genera
120 belonged to the healthy respiratory microbiome core (**Figure 1C** and **Table S5**). However, the relative
121 abundance of the healthy respiratory microbiota core was lower in critically ill patients than in healthy
122 controls (34% vs. 43%, respectively, p-value < 0.01, **Figure 1C**). More specifically, the relative
123 abundances of *Fusobacterium*, *Veillonella*, and *Prevotella* were lower in critically ill patients (p=0.03,
124 <0.001, and <0.001, respectively), while *Haemophilus* was significantly more abundant (p<0.001,
125 **Table S5**). *Pseudomonas* was observed in 53% of critically ill patients, with a mean relative abundance
126 reaching 5.3% (**Table S5**). The relative abundance of *Pseudomonas* in ICU patients was negatively
127 correlated with the relative abundance of the genera of the healthy respiratory core microbiota (**Figure**
128 **1D** for all sample types, and **Figure S1D** for BAL only). This series of analyses demonstrated that
129 critical illnesses are associated with respiratory dysbiosis.

130 We then examined whether the loss of healthy microbiota core was homogeneously observed
131 along the respiratory tract or specific to the sampling sites (OS, ETA, and BAL). Using beta diversity
132 that quantifies dissimilarities between samples, we observed that the three main contributors to variance
133 in the respiratory microbiota composition were the variables ‘Study’ (p<0.001, R²=0.12, **Figure S1E**),
134 the ‘Site of sampling’ (p<0.001, R²=0.008, **Figure S1F**), and then the medical condition ‘healthy vs.

135 critical illness'. Here, we found that BAL, ETA, and OS had significantly different beta diversity and,
136 as expected, that the alpha diversity decreased from OS to BAL (**Figure 1E**). At the genus level, only
137 two members of the healthy microbiome core (*Streptococcus*, *Prevotella*) were commonly found in
138 BAL, ETA, and OS, but their relative abundances varied with the site of sampling (**Figure 1F, and**
139 **Tables S6-8**). The replacement of the healthy core microbiome by *Pseudomonas* genera increased from
140 the upper airways (OS) to the alveolar spaces (BAL) in ICU patients (**Figure 1G and Figure S1G**). We
141 then determined which site offers the best compromise between clinical accessibility and accuracy. We
142 found that the richness of the ICU respiratory microbiome core, represented by the set size of the UpSet
143 plot, was higher in OS and ETA than in BAL. Still, as represented by the intersection size of the UpSet
144 plot, this signature was more frequently observed in BAL and ETA than in OS (**Figure 1H**). Given the
145 non-invasiveness and repeatability of ETA, we thus prioritized the development of microbiome
146 signatures in ETA samples.

147 ***ARDS microbiome score in ETA samples***

148 To develop a respiratory microbiota signature for ARDS, we selected critically ill participants with one
149 or more ETA samples collected during the first week of ICU hospitalization and ARDS outcome
150 available (number of ETA samples analyzed: 551). Out of the 450 patients included in four studies, 98
151 (22%) had ARDS at baseline or during the follow-up. We found that ETA samples from patients with
152 ARDS had a significantly different overall beta diversity than critically ill patients without ARDS
153 (**Figure 2A**). Since 'study' was the main contributor to the architecture of the respiratory microbiome
154 (**Figure S2A**), the study effect was treated as a blocking factor (i.e., confounder) for all the following
155 analyses. The alpha diversity of ETA was not statistically different between ARDS and no ARDS
156 patients (**Figures 2B-C**). When comparing patients with extreme Shannon index values (first quartile
157 vs last quartile values), the rate of ARDS was 21% in patients with low alpha diversity and 20% in those
158 with preserved alpha diversity ($P=1$, **Figure S2B**). The tracheal respiratory microbiome's composition
159 differed at the phylum and genus levels between patients with or without ARDS (**Figures 2D and S2C-**
160 **E**). Correlation network analysis showed different relationships between species among these groups of
161 patients (**Figures 2E-F**). Notably, the high relative abundance of *Streptococcus* correlated with the low
162 abundance of *Staphylococcus* in patients without ARDS.

163 Next, we selected the top features (i.e., genera) associated with ARDS using a Mann-Whitney U test
164 adjusted for 'study' and ANCOM-BC with adjusting for 'study'. With a p-value with a False Discovery
165 Rate (FDR corrected p-value) of 0.20, we identified a panel of 30 genera with different relative
166 abundance in ARDS and no ARDS microbiomes (**Figure 2G, Table S9**). We then employed a stacked
167 machine learning model consisting of spectral clustering and Fast and Frugal Trees (FFT) to define a
168 parsimonious predictive score using a minimal number of criteria. FFT-based modeling offered a simple,
169 3-factor decision tree that individually classifies samples as ARDS or no ARDS based on the presence
170 of *Staphylococcus*, *Ralstonia*, and *Enterococcus* (sensitivity = 92%, specificity = 63%, **Figure 2H**).
171 Shannon Index values and low alpha diversity subgroup (first quartile) were tested but not selected in

172 the FFT-based score. We applied a 10-fold cross-validation scheme to test the accuracy of the model
173 and calculated a mean-classification AUC of 0.751 (**Figure 2I**). Patients with no ARDS signature
174 defined by the FFT model had a higher probability of successful extubation than patients with ARDS
175 signature (Hazard Ratio (H.R.) 1.338, 95%CI 1.032-1.734, P=0.026, **Figure 2J**). The mortality rate in
176 patients with ARDS microbiome signature was 37% (49/132 patients) vs 27% (85/318 patients) in
177 patients with no ARDS signature (p=0.028), further supporting the clinical relevance of this FFT-based
178 classification.

179 As an exploratory analysis, we questioned the effect of the sampling time on the accuracy of the ARDS
180 signature. Thus, we built a risk index by calculating the sum of the relative abundances of taxa associated
181 with no ARDS minus the sum of the relative abundances associated with ARDS. The ARDS risk index
182 decreased when the relative abundance of ARDS-associated taxa increased. We observed that the risk
183 index values were not significantly different between early and late samples (ANOVA p-value =0.146,
184 **Figure S2F**). We also observed that the AUC of the model was 0.72 for samples collected during the
185 first week, 0.78 during the second week, and 0.73 afterwards (**Figure S2G**). This series of analyses
186 suggested that the developed ARDS microbiome signature was robust for samples collected during the
187 first three weeks of ICU hospitalization.

188 We also tested a score built by Classification Decision Tree, which can improve the model's accuracy
189 and interpretability, but usually uses more variables than FFT. The Classification Decision Tree-based
190 model included 10 factors but did not have significantly better accuracy than the parsimonious FFT-
191 based score (AUC = 0.789, sensitivity = 87.5%, specificity = 66.5%, **Figure S2H**).

192

193 ***Respiratory microbiome signature of ARDS in bronchoalveolar lavages did not significantly*** 194 ***outperform the ETA signature***

195 We tested the extrapolation of the ETA-based FFT model in the BAL sample dataset and observed a
196 decreased accuracy for ARDS classification (AUC = 0.68). We thus aimed to develop a BAL-specific
197 microbiome score associated with ARDS and repeated the approach described in Figure 2 in the cohort
198 of 252 participants from five studies with at least one BAL obtained during the first week of ICU
199 hospitalization (number of samples analyzed = 283, number of ARDS patients = 125/252, 50%). Even
200 after treating the study as a confounding factor for all analyses, we observed a statistically different
201 significant architecture of the respiratory microbiome between critically ill patients with or without
202 ARDS (**Figure S3A**). The alpha diversity was lower in samples from ARDS patients compared to no
203 ARDS patients (**Figure S3B-C**), and the composition of the BAL respiratory microbiome was different
204 at the phylum and genus levels between patients with or without ARDS (**Figure S3D-F**). The top
205 features selection identified a panel of 37 microbes that were differentially abundant between ARDS
206 and no ARDS BAL (**Figure S3G** and **Table S10** for taxa) and, as expected, different from those selected
207 in ETA (see **Figure 2G**). The FFT-based modeling offered a 3-factor decision tree based on the
208 abundances of *Flavobacterium*, *Veillonella*, and *Prostheco bacter* that predicted individual risk of ARDS

209 from the BAL microbiome with good accuracy (sensitivity = 85%, specificity = 76%, mean-
210 classification AUC of 0.81, **Figure S3H-I**). The model's overall accuracy was not significantly
211 improved when using the Classification Decision Tree method (AUC = 0.79, sensitivity = 75%,
212 specificity = 83%, **Figure S3J**). The accuracy of the BAL-specific microbiome signature for ARDS was
213 not significantly higher than the ETA-specific microbiome signature (0.751 vs 0.81, $p=0.514$ for mean
214 AUCs comparisons), supporting that the development of respiratory microbiome signatures in ETA
215 samples is a fair balance between accuracy and accessibility. Following the same strategy, we developed
216 a microbiome score in the OS dataset, which reached a low accuracy (AUC 0.65, data not shown),
217 further strengthening our decision to favor ETA samples for developing respiratory microbiome
218 signatures.

219

220 *HAP microbiome score*

221 We then developed a microbiome signature associated with HAP in a cohort of participants with at least
222 one ETA sample and a HAP outcome available. Out of the 738 patients included in 7 studies (number
223 of ETA samples = 955), 484 (65.6%) patients had HAP at baseline or during the follow-up. We observed
224 that ETA samples from critically ill patients with HAP had a significantly different overall architecture
225 than ETA from those without HAP, even after adjustment for the study effect (**Figure 3A** and **Figure**
226 **S4A** for the variability across the studies). The microbiome alpha diversity was lower in HAP than in
227 no-HAP samples collected in critically ill patients (**Figures 3B-C**). When comparing patients with
228 extreme Shannon index values (first versus last quartile values), the rate of HAP was 57% in patients
229 with low alpha diversity (first quartile) and 42% in those with preserved alpha diversity (fourth quartile,
230 $P=0.001$, **Figure S4B**). The composition of the ETA microbiome was different at the phylum level
231 between patients with or without HAP, with an increased relative abundance of Proteobacteria in HAP
232 patients (**Figure 3D**). *Streptococcus* and *Veillonella* relative frequencies were negatively correlated with
233 the relative frequency of *Pseudomonas* (**Figures 3E-F**).

234 The top feature selection approach identified a panel of 66 microbes that were differentially abundant
235 between HAP and no HAP samples (**Figure 3G** and **Table S11** for taxa). FFT-based modelling offered
236 a 4-factor decision tree associated with HAP classification (sensitivity = 73%, specificity = 71%, **Figure**
237 **3H**). Notably, HAP was associated with low relative abundances of *Streptococcus* and *Veillonella*, two
238 genera belonging to the healthy respiratory microbiome core. Shannon Index values and low alpha
239 diversity subgroup (first quartile) were tested but not selected in the FFT-based score. We applied a 10-
240 fold cross-validation scheme to test the accuracy of the FFT-based model for HAP classification and
241 calculated a mean-prediction AUC of 0.72 (**Figure 3I**). Patients with no HAP signature defined by the
242 FFT model had a higher probability of successful extubation than patients with a HAP signature (HR
243 1.505, 95%CI 1.16-1.952, $P=0.019$, **Figure 3J**). The mortality rate in patients with a HAP signature was
244 32% (68/213 patients) vs. 29% (95/326 patients) in those with no HAP microbiome signature ($p=0.49$).

245 We tested this model in BAL and observed a decreased accuracy for HAP classification (AUC = 0.61),
246 which did not support the extrapolation of this FFT model in distal samples.

247 As an exploratory analysis, the accuracy of the HAP microbiome score estimated by the Classification
248 Decision Tree was 0.67 (sensitivity = 56%, specificity = 71%, **Figure S4C**). We also questioned the
249 effect of the sampling time on the accuracy of the HAP signature. Thus, we built a risk index for HAP
250 by calculating the sum of the relative abundances of taxa associated with no HAP minus the sum of the
251 relative abundances associated with HAP. The HAP risk index decreased over time (ANOVA p-value
252 = 0.002, **Figure S4D**), demonstrating that the total relative abundance of taxa associated with HAP
253 increased after two weeks in ICU. In a subgroup analysis based on sample timing, the AUC of the FFT-
254 based model for HAP was 0.72 during the first week, 0.72 during the second week, and 0.66 beyond
255 two weeks (**Figure S4E**), demonstrating that the HAP signature accuracy decreased after two weeks in
256 ICU.

257

258 **Early successful extubation microbiome signature**

259 The diagnoses of ARDS and HAP are faced with challenges in clinical practice, due to inherent
260 subjectivity, especially with regards to radiographic data interpretation for ARDS, as well as sensitivity
261 and specificity limitations of microbiology for HAP. As such, it is important to examine microbiome
262 signatures independent of ARDS/HAP diagnoses that can predict clinical outcome.^{2,6} Successful
263 extubation (i.e., liberation from MV) is an important patient-centered outcome. Therefore, we tested
264 whether defining a microbiota signature could predict the time-to-successful extubation in critically ill
265 patients. Among the ICU patient population with ETA samples, all samples had been collected before
266 weaning from MV (n=254), and the median time to successful extubation was 10 (4-11) days since ICU
267 admission. We thus developed a score to predict early (< 4 days, first quartile) vs. late (> ten days, fourth
268 quartile) successful extubation and found 18 features associated with these outcomes (**Figure 4A, Table**
269 **S12**). The FFT-based modeling offered a 3-factor decision tree for classification as early successful
270 extubation based on the relative abundances of *Lactobacilliales*, *Rothia*, and *Streptococcus* (sensitivity
271 = 85%, specificity = 60%, **Figure 4B**). We applied a 10-fold cross-validation scheme to test the accuracy
272 of the FFT-based prediction model for early successful extubation and calculated a mean-prediction
273 AUC of 0.727 (**Figure 4C**). We tested the ETA-based model in BAL sample datasets (n=154 patients,
274 median MV duration 10 days) and observed a similar accuracy for time to extubation classification
275 (AUC 0.71). Further supporting the medical relevance of this score, the mortality rate in patients with
276 an early successful extubation microbiome signature was 23% (49/217 patients) vs 36% (85/233
277 patients) in those with prolonged MV signature (p=0.001).

278 As an exploratory analysis, the Classification Decision Tree-based model also included three factors
279 (*Lactobacilliales*, *Rothia*, and *Haemophilus*) with increased accuracy (AUC 0.882, sensitivity = 82%,
280 specificity = 90%, **Figure S5A**). To test the impact of the early successful extubation definition, we
281 built a risk index by calculating the sum of the relative abundances of taxa associated with early

282 extubation minus the sum of the relative abundances associated with prolonged MV. The risk index thus
283 varied from -1 (samples rich in taxa associated with prolonged MV) to +1 (samples rich in taxa
284 associated with early extubation), and had a fair accuracy for predicting prolonged MV (Figure S5B).
285 We observed that the risk index of samples collected during the first week of hospitalization was higher
286 than those collected during the second week or after (**Figure S5C**). This observation suggests that the
287 early successful extubation signature can be useful in ICU populations with a median duration of MV
288 of close to one week, routinely reported in ARDS (median 8 days)² and HAP (median 7 days)³ patients.
289

290 **External validation of the microbiome signatures of ARDS, HAP, and early successful extubation**

291 Finally, we aimed to validate the accuracy of these scores in a prospective independent cohort of ICU
292 patients. We analyzed 277 ETA samples collected on days 0, 4, and 7 after ICU admission in a cohort
293 of 136 brain-injured patients requiring invasive MV hospitalized in one French hospital. The study
294 population characteristics are described in **Table S13**. The rates of ARDS and HAP were 38% and 51%,
295 respectively, and all cases of ARDS were related to severe HAP.

296 We confirmed that the respiratory microbiome architecture differed between patients with or without
297 ARDS, patients with or without HAP, and patients with or without early successful extubation (**Figure**
298 **5A-F**). The ETA-based microbiome FFT scores for ARDS, HAP, and early successful extubation
299 performed reasonably in this independent cohort of severely brain-injured patients (AUC= 0.75, 0.70,
300 and 0.68, respectively, **Figure 5G-I**). Finally, we observed that patients with ARDS, HAP or prolonged
301 MV microbiome signatures based on the 3 or 4 criteria FFT had longer times to successful extubation
302 than patients with no signatures (Hazard Ratio 1.56 (95CI% 1.07-2.27), 1.51 (95%CI 1.02-2.23) and
303 1.50 (95%CI 1.03-2.18), respectively) (**Figure 5J-L**).

304

305 **DISCUSSION**

306 While the clinical presentations of HAP and ARDS frequently overlap, we defined and validated
307 two different respiratory microbiome signatures: HAP signature was mainly characterized by a low
308 abundance of the healthy microbiome. In contrast, the ARDS signature consisted of high levels of
309 pathogenic bacteria. This observation can substantially impact the diagnosis of these two conditions and
310 suggest two different types of microbiome manipulation to treat them: restoration of commensal bacteria
311 to prevent and treat HAP²⁸, and aggressively targeting pathogens with therapies that do not alter
312 commensal in patients with ARDS (e.g., genera-specific antibodies²⁹).

313 The administration of probiotics designed to restore the gut microbiome failed to prevent HAP
314 and reduce MV in critically ill patients^{30,31}. Developing and validating specific modifications in the
315 respiratory microbiota composition can aid in identifying bacterial consortia that can be administrated
316 to prevent or treat respiratory complications³². Association of HAP with a low abundance of
317 *Streptococcus* is of particular interest since the secretion of a bacterial peptidoglycan hydrolase by
318 *Streptococcus* taxa decreases the severity of gram-negative mucosal infection³³, and lung colonization

319 with *Streptococcus pneumoniae* enhanced alveolar macrophages responses to bacteria ³⁴. From a
320 therapeutic perspective, bacterial administration directly into the lungs of patients can be challenging
321 and potentially poorly tolerated ^{35,36}. Another approach could be directly administering the bacterial-
322 derived metabolites that regulate the immune cell response ¹² and affect pneumonia outcomes ³⁷. Future
323 investigations of respiratory microbiomes should aim at deep sequencing (i.e., shotgun metagenomic
324 and metabolomic datasets) to understand the functional consequences of dysbiosis better.³⁸

325 A high relative abundance of *Rothia*, a gram-positive coccus member of the family
326 Micrococcaceae, which is considered part of the normal microflora of the upper respiratory tract ³⁹, was
327 associated with absence of HAP and short duration of MV but counterintuitively with ARDS. Several
328 interpretations can be discussed to explain these findings. First, ARDS is a heterogenous condition,
329 *Rothia* abundance could define an ARDS sub-group with favourable outcomes (no superinfection and
330 rapid weaning from MV). Moreover, given the nature of the data (16sRNA sequencing), we cannot
331 exclude the possibility of differences in *Rothia* sub-species composition between favorable and
332 unfavorable outcomes.

333 Given the high inter-person variability of the respiratory microbiome composition, it is unlikely
334 that probiotics will benefit all critically ill patients. The development and validation of frugal scores,
335 using a limited number of bacteria, that predict individual outcomes is thus a significant deliverable of
336 this study. However, even if real-time metagenomics enables rapid pathogen identifications in bacterial
337 pneumonia ⁴⁰, it cannot currently be feasibly implemented at the bedside. Standardized rapid multiplex
338 PCR panels accurately identify tens of bacteria in respiratory fluids in HAP patients and deliver results
339 within a few hours after sampling ⁴¹⁻⁴³. While most of the current rapid biomolecular tests aim to identify
340 pathogens, monitoring the healthy commensal abundance could be included in these biomolecular tests
341 to predict individual responses and guide treatment at the bedside.

342 Developing reliable respiratory microbiome signatures required pooling data from various
343 studies due to the limited size of reported cohorts. The heterogeneity in the included studies, regarding
344 geographic location, inclusion criteria, sampling methods, and ARDS and HAP definitions, was an
345 important consideration in our analyses. To limit the risk of bias in our meta-analysis, we had to control
346 for study variability by adjusting or blocking study effects in the analyses. Despite this methodological
347 approach, the sampling heterogeneity potentially biased the comparison between BAL and ETA score
348 accuracies because differences in study populations cannot be definitively excluded. Several exploratory
349 analyses were performed to test the score robustness. First, we found an enrichment of the ICU
350 microbiome signature from the alveolar spaces to the upper airways, suggesting an anatomical
351 continuum of the pathophysiological mechanism. Second, we observed that the accuracy of the score
352 developed in the BAL dataset did not outperform the ETA-based score. Third, the validation cohort
353 demonstrated the robustness of our statistical approach and supported the extrapolation of the defined
354 signatures in other ICUs. This series of analyses suggested that the signatures are robust in BAL and
355 ETA, and we proposed that if BAL had the best accuracy for ARDS prediction, scores developed in

356 ETA are acceptable alternatives with potential higher applicability in clinical practice. However, the
357 external validation was only performed in ETA samples, and caution before using ETA-based signatures
358 on BAL samples is required.

359 When comparing samples from healthy individuals and ICU patients, the analysis of microbial
360 beta diversity showed that study heterogeneity had a larger effect on overall microbiome composition
361 than the condition in our pooled data. This limit has also been reported in meta-analyses that assessed
362 the association of gut microbial signatures with colorectal cancer ⁴⁴ or immune checkpoint inhibitor
363 response in advanced melanoma ⁴⁵. The early sample timing observed in our study can explain that the
364 condition's effect was lower than the study's effect. Indeed, we observed that the alpha diversity
365 decreased over time, and early sampling may underestimate the association between respiratory
366 dysbiosis and outcomes, especially for patients who are not liberated early during MV.

367 Our study has several limitations. Firstly, the large sample size of the pooled analysis population
368 allowed the identification of statistically significant differences which may be of questionable clinical
369 significance. Moreover, given the observational nature of our data, we can not conclude if microbiota
370 dysbiosis contributes to ARDS and HAP or if ARDS/HAP cause microbiota alterations. Secondly, we
371 could not exclude the possibility of confounding via clinical exposures, including antibiotics. We
372 deliberately did not adjust our analyses for antibiotic exposure, given that respiratory dysbiosis may
373 mediate clinical effects attributable to antibiotics and the risk of confounding by indication. Recent
374 studies revealed associations between the administration of anti-anaerobic antibiotics and adverse
375 clinical outcomes among mechanically ventilated patients ^{46,47}. If, as speculated, this association is
376 mediated by antibiotic effects on the microbiome, adjusting for antibiotics would inappropriately
377 obscure an important causal relationship. Thirdly, our study samples were collected early, most during
378 the first week of hospitalization, and the signature accuracies could decrease after two weeks. Finally,
379 the external validation was performed in a homogeneous population of severely brain-injured patients,
380 potentially limiting the extrapolation of our findings to other ICU populations. However, the discovery
381 cohort was built by pooling data from heterogeneous populations (from cardiac arrest to post-operative
382 care). Since none of these studies has included brain-injured patients, the chosen validation cohort still
383 increases the spectrum of critical illness investigated in our meta-analysis.

384 Our findings are pivotal for describing specific ARDS and HAP respiratory microbiome
385 signatures and associating these respiratory microbiome profiles with unfavourable clinical outcomes.
386 The 3- to 4-factor decision trees increased our understanding of the pathophysiological causes of these
387 conditions and could form the basis of diagnostic assays to test for clinical implementation. Our
388 evidence synthesis can inform future work that aims to mechanistically investigate the contribution of
389 the respiratory microbiome on lung immunity and inflammation and help to define promising
390 therapeutic targets for specific microbiome modulation in critically ill patients.

391

392 **ONLINE METHODS**

393 ***Search strategy for available respiratory microbiome data.***

394 We followed the recommended Preferred Reporting Items for Systematic Reviews and Meta-analyses
395 (PRISMA) guidelines to select the articles. We searched in MEDLINE, EMBASE, Web of Science, and
396 Scopus databases for articles published in peer-reviewed journals written in English and indexed until
397 May 2022. We combined several search terms, including microbiota, microbiome, microbial
398 community, gut microbial composition, respiratory microbiome, lung microbiome, metagenomics,
399 lower respiratory tract, oropharyngeal tract, intensive care unit, mechanical ventilation, acute respiratory
400 distress syndrome, ventilator-associated pneumonia.

401 **MEDLINE and EMBASE**

402 #1. (microbiom* or microbiot* or microflor* or respiratory flor* or v or microbial respiratory com* or
403 lower respiratory tract flor* or oropharyngeal flor*).mp. (mp=title, abstract, heading word, original title,
404 keyword, floating subheading word, candidate term word)

405 #2. (acute respiratory distress syndrome).mp. (mp=title, abstract, heading word, drug trade name,
406 original title, drug manufacturer, keyword, floating subheading word, candidate term word)

407 #2. (ventilator-associated pneumonia).mp. (mp=title, abstract, heading word, drug trade name, original
408 title, drug manufacturer, keyword, floating subheading word, candidate term word)

409 #3. 1 and 2

410 #4. limit 3 to English language

411 #5. limit 4 to article

412 **Web of Science**

413 #5 #2 AND #1 Refined by: DOCUMENT TYPES: (ARTICLE) AND LANGUAGES: (ENGLISH)
414 DocType=All document types; Language=All languages;

415 #4 #2 AND #1 Refined by: DOCUMENT TYPES: (ARTICLE) DocType=All document types;
416 Language=All languages;

417 #3 #2 AND #1 DocType=All document types; Language=All languages;

418 #2 TOPIC: (acute respiratory distress syndrome) DocType=All document types; Language=All
419 languages;

420 #2 TOPIC: (ventilator-associated pneumonia) DocType=All document types; Language=All
421 languages;

422 #1 TOPIC: (microbiom* OR microbiot* OR microflor* OR respiratory flor* OR respiratory flor* OR
423 microbial respiratory com* OR lower respiratory tract flor* OR oropharyngeal flor*) DocType=All
424 document types; Language=All languages;

425 **SCOPUS**

426 (TITLE-ABS-KEY (microbiom* OR microbiot* OR microflor* OR (lung AND flor*) OR (respiratory
427 AND flor*) OR (lower respiratory tract AND flor*) OR (oropharyngeal AND flor*) OR (or microbial
428 respiratoty com*)) AND (TITLE-ABS-KEY (acute respiratory distress syndrome)) AND (LIMIT-TO
429 (DOCTYPE, "ar")) AND (LIMIT-TO (LANGUAGE, "English"))

430 (TITLE-ABS-KEY (microbiom* OR microbot* OR microflor* OR OR (lung AND flor*) OR
431 (respiratory AND flor*) OR (lower respiratory tract AND flor*) OR (oropharyngeal AND flor*) OR (or
432 microbial respiratoty com*)) AND (TITLE-ABS-KEY (ventilator-associated pneumonia)) AND
433 (LIMIT-TO (DOCTYPE, "ar")) AND (LIMIT-TO (LANGUAGE, "English"))

434

435 ***Study inclusion criteria in the meta-analyses***

436 We included observational (cross-sectional, case-control, and cohort designs) and interventional studies
437 involving adult critically ill adults (age 18 years or older) that reported 16S rRNA amplicon sequencing
438 respiratory microbiota data and described ARDS and/or HAP occurrence during intensive care unit
439 hospitalization. We also included studies reporting the respiratory composition of healthy adults to serve
440 as controls.

441

442 ***Outcomes***

443 We assessed differences in the respiratory microbiome in terms of alpha and beta diversity and
444 taxonomic composition in the following clinical group comparisons: in critically ill patients hospitalized
445 in ICU compared to healthy individuals; in patients with ARDS compared to patients without ARDS;
446 in patients with HAP, compared to patients without HAP; and in patients with late successful extubation
447 compared to patients with early successful extubation defined as the highest versus lowest quartile of
448 MV duration in survivors. When investigating the time to weaning from MV, mortality was treated by
449 censoring the observed duration of MV. We applied the definitions of ARDS and HAP used in the
450 original studies. Day 0 was defined as in-ICU admission. Microbiome profiles of patients with HAP and
451 ARDS were independently used to develop both microbiome signatures.

452

453 ***Study selection and data extraction***

454 All titles and abstracts found by the search strategy reported in above were screened for relevance by
455 E.M. and QLB. Titles and abstracts were then retrieved in full and evaluated for inclusion eligibility in
456 the meta-analysis by E.M. and Q.L.B. Eligibility results were compared and discussed between QLB,
457 AR, and E.M. to retain 20 studies with microbiome data from healthy individuals and ICU patients. Raw
458 sequencing data (i.e., FATSQ files) were then uploaded from the specified database
459 (<https://www.ebi.ac.uk/> and <https://www.ncbi.nlm.nih.gov/sra>) and metadata were requested from the
460 corresponding authors. Two studies analyzed ICU microbiome but did not report ARDS, HAP or MV
461 duration. Moreover, despite contacting the corresponding authors, we could not access data from one
462 study ⁴⁸, leaving 17 studies for the meta-analysis (13 studies with microbiome from ICU patients, 4
463 studies with microbiome from healthy individuals).

464

465 ***Microbiota analyses***

466 Sequences were preprocessed, quality-filtered, and analyzed using QIIME 2 (2019.10 release)
467 (<https://qiime2.org/>)⁴⁹. QIIME 2 computes error-corrected amplicon sequence variants (ASV) for
468 Illumina read sequences. We used QIIME 2 in combination with its Deblur plugin ⁵¹. Raw reads were
469 imported into a QIIME 2 artifact before merging paired-end reads and quality filtering. Reads were then
470 denoised using the “deblur denoise-16S” command and trimmed based on demux-summary.qzv.
471 Representative sequences and their abundances were extracted by feature-table ⁵¹. A naive Bayes
472 classifier was fitted with 16S rRNA gene sequences extracted from Greengenes version 13_8 ⁵². ASVs
473 classified as mitochondria or chloroplasts were excluded from further analysis. Compositions of
474 microbiota communities were summarized by proportions at different taxonomy levels, including genus,
475 family, order, class, and phylum ranks. We then analyzed the respiratory microbiome diversity. First,
476 we assessed alpha diversity that summarizes the distribution of ASV in each sample into a single number
477 based on ASV richness and evenness, using the Shannon index and the number of observed genera. We
478 also assessed the beta diversity that describes the differences in composition between samples by
479 calculating the Bray-Curtis distance. Tests for categorical differences in beta diversity were performed
480 using PERMANOVA as implemented in R’s vegan package (R package version 2.6-4). Alpha diversity
481 measures (observed ASV and Shannon diversity) were calculated from the ASV tables collapsed at the
482 genus level, using diversity as implemented in R’s vegan package (R package version 2.6-4). We next
483 plotted network correlation plot of the core respiratory microbiome in the respiratory samples collected
484 in critically ill patients, using a cut-off for prevalence (presence of a genus in at least 50% of the cohort)
485 and Spearman’s correlation analysis. We compared samples collected in patients with or without ARDS,
486 and samples collected in patients with or without HAP using a Mann-Whitney U test blocked for ‘study’
487 and ANCOM-BC with adjusting for ‘study’ A p-value with a False Discovery Rate (FDR corrected p-
488 value) of 0.20 was considered significant. To aid clinical decision-making, we fitted a Fast and Frugal
489 Tree (FFT) to predict risk groups accurately ⁵³⁻⁵⁵. FFTs are simpler versions of decision trees and have
490 been shown to perform competitively with random forests and to facilitate biologic interrogation by
491 decreasing the number of key features. Data were processed with default settings except when stated
492 otherwise.

493

494 ***Independent cohort for external validation***

495 ***Human subjects and human samples***

496 Bioresources: IBIS (cohort), Nantes, France. Patients were enrolled from 31st January 2017 to 5th May
497 2020 in two French Surgical Intensive Care Units of one university hospital (Nantes, France). The
498 collection of human samples has been declared to the French Ministry of Health (Programme de
499 recherche “Immunologie”, DC2014-2206, authorization renewed DC-2017-2987), and was approved by
500 the Comite de Protection des Personnes Ouest IV (7/04/2015 and 08/10/2020). Written informed consent
501 from a next-of-kin was required for enrolment. Retrospective consent was obtained from patients when
502 possible. Appropriate consent was obtained for release of information from deceased individuals.

503 Inclusion criteria were brain injury (Glasgow Coma Scale below or equal to 12 and abnormal brain-CT
504 scan) and receiving invasive MV. Exclusion criteria were cancer in the previous five years,
505 immunosuppressive drugs, and pregnancy. All patients were clinically followed up for 90 days.
506 Endotracheal aspirates were collected on days 0, 4, and 7 after ICU admission, frozen at -80c, and stored
507 until sequencing (see study protocol in Supplementary files).

508

509 ***Sequencing and analysis of ETA samples from the IBIS cohort.***

510 *Specimen processing:* Specimens were centrifuged (30 minutes at 15,000 G) and the resulting pellet was
511 used for DNA isolation. Pellets were resuspended in 360µl ATL buffer (Qiagen DNeasy Blood & Tissue
512 kit). Sterile laboratory water and AE buffer used in DNA isolation were collected and analyzed as
513 potential sources of contamination, as were extraction controls (empty isolation tubes) and blank
514 sequencing wells.

515 *Bacterial DNA isolation:* Genomic DNA was extracted from mini-BAL pellets (Qiagen DNeasy Blood
516 & Tissue kit, Qiagen, Hilden, Germany) using a modified protocol previously demonstrated to isolate
517 bacterial DNA[1]. Sterile laboratory water and AE buffer used in DNA isolation were collected and
518 analyzed as potential sources of contamination. Specimens were processed in a randomized order to
519 minimize the risk of false pattern formation due to reagent contamination[2].

520 *16s rRNA gene sequencing:* The V4 region of the 16s rRNA gene was amplified using published
521 primers[3] and the dual-indexing sequencing strategy developed by the laboratory of Patrick D.
522 Schloss[4]. Sequencing was performed using the Illumina MiSeq platform (San Diego, CA), using a
523 MiSeq Reagent Kit V2 (500 cycles), according to the manufacturer's instructions with modifications
524 found in the Schloss SOP[5]. Accuprime High Fidelity Taq was used in place of Accuprime Pfx
525 SuperMix. Primary PCR cycling conditions were 95°C for two minutes, followed by 20 cycles of
526 touchdown PCR (95°C 20 seconds, 60°C 20 seconds and decreasing 0.3 degrees each cycle, 72°C 5
527 minutes), then 20 cycles of standard PCR (95°C for 20 seconds, 55°C for 15 seconds, and 72°C for 5
528 minutes), and finished with 72°C for 10 minutes.

529 *Bacterial DNA quantification:* Bacterial DNA was quantified using a QX200 Droplet Digital PCR
530 System (BioRad, Hercules, CA). Primers and cycling conditions were performed according to a
531 previously published protocol[6]. Specifically, primers were 5'- GCAGGCCTAACACATGCAAGTC-
532 3' (63F) and 5'- CTGCTGCCTCCCGTAGGAGT-3' (355R). The cycling protocol was 1 cycle at 95°C
533 for 5 minutes, 40 cycles at 95°C for 15 seconds and 60°C for 1 minute, 1 cycle at 4°C for 5 minutes,
534 and 1 cycle at 90°C for 5 minutes all at a ramp rate of 2°C/second. The BioRad C1000 Touch Thermal
535 Cycler was used for PCR cycling. Droplets were quantified using the Bio-Rad Quantisoft software. Two
536 replicates were used per sample. No-template control specimens were used and were run alongside mini-
537 BAL specimens.

538 *Statistical analysis:* We used the same method for the prospective cohort analysis as for the meta-
539 analysis.⁵⁰⁵⁰

540 **Scripts for the microbiome analysis**

541 We described here all the custom code used for the analyses.

542 *Alpha diversity*

543 Alpha diversity measures (observed ASV and Shannon diversity) were calculated from the ASV tables
544 collapsed at the genus level, using diversity as implemented in R's vegan package (R package version
545 2.6-4). Alpha diversity was expressed as the Shannon index for normalized numbers of sequences for
546 each sample. Rarefaction was done by random subsampling with 1043 reads as threshold. Data were
547 processed with default settings except when stated otherwise.

```
548 #example for Shannon index in ARDS samples vs non-ARDS samples
549 #m : mapping that contains the metadata of the samples
550 #x: ASV table collapsed at genus level
551 library(vegan)
552 x <- x [(rownames(m)) , ]
553 SHA <- as.data.frame(diversity(x, index = "shannon"))
554 colnames(SHA) <- c('shannon')
555 wilcox.test(SHA$shannon ~ m$ARDS)
556 #plot alpha diversity
557 pdf('alpha_div_SHA.pdf,width=4,height=4);
558 e <- ggplot(SHA, aes(x = ICU, y = OS))
559 e + geom_violin(aes(fill = ICU)) +
560   geom_boxplot(width=0.05) +
561   scale_fill_manual(values = body_col])+theme_cowplot(font_size = 7) +
562   theme(axis.text.x = element_text(color=NA), axis.text.y = element_text(color=NA))
563 dev.off()
564
```

565 *Beta diversity*

566 Tests for categorical differences in beta diversity were performed using PERMANOVA as implemented
567 in R's vegan package (Bray-Curtis distances with permutational analysis of variance [permanova] at
568 1000 permutations). β diversity was summarised using principal coordinate (PCo) analysis of Bray-
569 Curtis distances, as they represent most features of β diversity. The R script used for beta diversity is
570 described below:

```
571 #example for betadiversity in ARDS samples vs non-ARDS samples
572 #m : mapping that contains the metadata of the samples
573 #x: ASV table collapsed at genus level
574 library(vegan)
575 library(ape)
576 beta_table <- as.matrix(vegdist(x), method = "bray", na.rm = F)
577 PCOA <- pcoa(beta_table)$vectors
578 var_exp <- pcoa(beta_table)$values
579 #Run stats for diff. centroids
580 beta_dist = as.dist(beta_table)
581 length(beta_dist)
582 #Run permanova
583 ad = adonis(beta_dist ~ m$ARDS, permutations=999)
584 p_val <- ad$aov.tab[1,6]
585 r_sq <- ad$aov.tab[1,5]
586 #Run Stats for diff. dispersion
```

```

587 beta_out <- betadisper(beta_dist, m$ARDS)
588 p_val_disp <- permutest(beta_out)$stab[1, 6]
589 #stats sur les coordinates
590 PCOA <- PCOA[rownames(m),]
591 wilcox.test(PCOA[,1] ~ m$ARDS)
592 wilcox.test(PCOA[,2] ~ m$ARDS)
593 #plot betadiversity PCoA
594 for(i in 1:ncol(PCOA)){
595   colnames(PCOA)[i] <- paste("PC",i, sep="")
596 }
597 PCOA <- cbind(PCOA, rownames(PCOA))
598 colnames(PCOA)[ncol(PCOA)] <- "SampleID"
599 m <- cbind(m, rownames(PCOA))
600 m <- data.frame(lapply(m, as.character), stringsAsFactors=FALSE)
601 colnames(m)[ncol(m)] <- "SampleID"
602 PCOA <- merge(PCOA, m, by="SampleID")
603 PCOA$PC1 <- as.numeric(as.character(PCOA$PC1))
604 PCOA$PC2 <- as.numeric(as.character(PCOA$PC2))
605 PCOA$PC3 <- as.numeric(as.character(PCOA$PC3))
606 PCOA$PC4 <- as.numeric(as.character(PCOA$PC4))
607
608 #Make PCoA plot
609 body_PCOA <- ggplot(PCOA) +
610   geom_point(size = 2, alpha=0.65, aes_string(x = "PC1", y = "PC2", color = "ARDS")) +
611   scale_color_manual(values=body_cols) +
612   theme_cowplot(font_size = 7) +
613   guides(color=F) +
614   annotate("text", x=-0.45, y=-0.2, label= paste("P=", p_val), size=2) +
615   annotate("text", x=-0.45, y=-0.25, label= paste("R2=", round(r_sq, digits=3)), size=2) +
616   labs(x="", y="") +
617   theme(axis.text.x = element_text(color=NA), axis.text.y = element_text(color=NA))
618 #Make boxplot of PCs
619 PC1_boxes <- ggplot(PCOA) +
620   geom_boxplot(aes_string(x = factor(PCOA$ARDS, levels=c("ARDS", "no_ARDS")), y = "PC1", fill
621 = "ARDS")) +
622   scale_fill_manual(values=body_cols) +
623   theme_cowplot(font_size = 7) +
624   guides(fill=F)+
625   coord_flip() +
626   labs(x="", y= paste("PC1 (", round(var_exp$Relative_eig[1], digits=3)*100, "%)", sep=""))
627
628 PC2_boxes <- ggplot(PCOA) +
629   geom_boxplot(aes_string(x =factor(PCOA$ARDS, levels=c("ARDS", "no_ARDS")), y = "PC2", fill
630 = "ARDS")) +
631   scale_fill_manual(values=body_cols) +
632   theme_cowplot(font_size = 7) +
633   guides(fill=F) +
634   labs(x = "", y= paste("PC2 (", round(var_exp$Relative_eig[2], digits=3)*100, "%)", sep="")) +
635   theme(axis.text.x = element_text(color=NA))
636
637 #Compile the PCoA and boxes
638 top2 <- plot_grid(PC2_boxes, body_PCOA, ncol=2, rel_widths=c(0.3, 1))
639 bottom2 <- plot_grid(NULL, PC1_boxes, ncol=2, rel_widths=c(0.3, 1))
640 together2 <- plot_grid(top2, bottom2, nrow=2, rel_heights=c(1, 0.3))
641 pdf("betadiv.pdf",width=7,height=3.5);

```

```
642 together2
643 dev.off()
644
```

645 *Correlation network*

646 We plotted network correlation plot of the core respiratory microbiome in the respiratory samples
647 collected in critically ill patients (including samples collected at BAL, ETA, and OS), using a cut-off
648 for prevalence (presence of a genus in at least 50% of the cohort) and Spearman's correlation analysis.
649 We used Cytoscape⁴⁹ to plot the results of the spearman correlation, and all correlations between the
650 most prevalent genera were plotted. We plotted all the respiratory microbiome, combining BAL, ETA
651 and OS samples, and also we plotted separately BAL, ETA or OS samples as detailed in the Figure 1.

652 The R script is described below:

```
653 library(circlize)
654 library(scales)
655 library(reshape)
656 M <- cor(x)
657 dim(M)
658 PAIRS <- melt(M)
659 PAIRS_2 <- PAIRS[!(PAIRS$value=="1"),]
660 head(PAIRS_2)
661 pdf("chordDiagram.pdf")
662 chordDiagram(PAIRS_2, annotationTrack = c("grid", "name"), col = pal, preAllocateTracks =
663 list(track.height = 0.3),
664         order = names
665         , grid.col = setNames(body_col)
666         , names))
667 draw(lgd, x = unit(1, "npc"), y = unit(1, "npc"), just = c("right", "top"))
668 dev.off()
669
```

670 The 3 shared genera (Figure S1G, *Streptococcus*, *Prevotella*, and *Pseudomonas*), were compared
671 assessed by Kruskal-Wallis and Dunn's tests.

```
672 library(FSA)
673 kruskal.test(x[,5] ~ meta$host)
674 PT = dunnTest(x[,5] ~ meta$host, method="fdr")
675
```

676 *The upset plot*

677 UpSetR generates static UpSet plots⁴⁹ The UpSet technique visualizes set intersections in a matrix
678 layout and introduces aggregates based on groupings and queries. The matrix layout enables the
679 effective representation of associated data, such as the number of elements in the aggregates and
680 intersections (here features in BAL, ETA and OS), as well as additional summary statistics derived from
681 subset or element attributes (here for example the common features in BAL, ETA and OS, n = 3).

682

683 *Mann-Whitney U test blocked for 'study'*

684 For the association studies, all timepoints were included. Especially for the prospective cohort, the 3
685 timepoints were used for the analyses. We used the package R coin.⁵⁶

```
686 # returns p-values, q-values (FDR corrected p-value),
```

```

687 load library(coin)
688 "differentiation.test" <- function (x,category, alpha=0.20, parametric=FALSE,
689     include.subset=FALSE){
690     category <- as.factor(as.character(category))
691     if(length(unique(category)) < 2) stop('Category only has one level')
692     if(parametric){
693         pvals <- apply(x,2, function(taxon){
694             if(var(taxon) == 0){
695                 NA
696             } else {
697                 summary(lm(taxon~category))[[4]][2,4]
698             }
699         })
700     stats <- apply(x,2, function(taxon){
701         if(var(taxon) == 0){
702             NA
703         } else {
704             summary(lm(taxon~category))[[4]][2,1]
705         }
706     })
707     } else {
708         if(length(levels(category)) == 2){
709             ix1 <- category == levels(category)[1]
710             ix2 <- category == levels(category)[2]
711             pvals <- apply(otus,2,function(xx) pvalue(wilcox_test(xx ~ as.factor(m$Study))))
712             stats <- apply(x,2,function(taxon)
713 wilcox.test(taxon[ix1],taxon[ix2],exact=FALSE)$statistic)
714             } else {
715             pvals <- apply(otus,2,function(xx) pvalue(wilcox_test(xx ~ as.factor(m$Study))))
716             stats <- apply(x,2,function(taxon) kruskal.test(taxon ~ category)$statistic)
717             }
718         }
719         na.ix <- is.na(pvals)
720
721         adj.pvals <- rep(NA,length(pvals))
722         names(adj.pvals) <- names(pvals)
723         adj.pvals[!na.ix] <- p.adjust(pvals[!na.ix],'FDR')
724         keep.ix <- adj.pvals < alpha
725         keep.ix[is.na(keep.ix)] <- FALSE
726         if(!any(keep.ix)) stop('select.features failed to find any features.')
727
728         # add stars to column names based on significance
729         annotations <- colnames(x)
730         thresholds <- c(.05, .01, .001, .0001)
731         for(i in seq_along(thresholds)){
732
733             star.ix <- adj.pvals[!na.ix] <= thresholds[i]
734
735             if(any(star.ix)){
736                 for(j in which(star.ix)){
737                     annotations[!na.ix][j] <- paste(annotations[!na.ix][j], '*', sep='')
738                 }
739             }
740         }
741         result <- list()

```

```

742     result$annotations <- annotations
743     result$features <- which(keep.ix)
744     result$qvalues <- adj.pvals
745     result$pvalues <- pvals
746     result$stats <- stats
747
748     # classwise means
749     result$classwise.means <- t(apply(x,2,function(xx) sapply(split(xx,category),mean)))
750     colnames(result$classwise.means) <- sprintf('%s_mean',colnames(result$classwise.means))
751
752     if(include.subset){
753         result$subset <- x[,keep.ix,drop=F]
754         colnames(result$subset) <- annotations[keep.ix]
755     }
756
757
758     return(result)
759 }
760
761 # saves list of results from differentiation.test to file (or prints)
762 "write.differentiation.test.results" <- function(results, filename='differentiated.features.txt'){
763     if(!is.null(filename)){
764         scipen.save <- options('scipen')
765         options(scipen=20)
766         hits <- cbind(results$pvalues, results$qvalues)
767         hits <- cbind(hits, results$classwise.means)
768         colnames(hits)[1:2] <- c('pvalue','qvalue')
769         hits <- hits[!is.na(hits[,1]),]
770         hits <- hits[order(hits[,1]),]
771         sink(filename)
772         cat('Feature\t')
773         write.table(hits,quote=F,sep='\t')
774         sink(NULL)
775         options(scipen=scipen.save)
776     }
777 }
778
779 ANCOM-BC with adjusting for 'study.'
780 The current code implements ANCOM-BC in cross-sectional and longitudinal datasets while allowing
781 the use of covariates [13]. We applied a methodology called Analysis of Compositions of Microbiomes
782 with Bias Correction (ANCOM-BC), which estimates the unknown sampling fractions and corrects the
783 bias induced by their differences among samples. The relative abundance data are modeled using a linear
784 regression framework, and the method provides statistically valid test with appropriate p-values and
785 controls the False Discovery Rate (FDR).
786
787 #load the files
788 otu_mat<- read_excel("OTU_table.xlsx")
789 tax_mat<- read_excel("Taxonomy_table.xlsx")
790 samples_df <- read_excel("mapping_file.xlsx")
791
792 #define the row names from the otu column

```

```

793 otu_mat <- otu_mat %>%
794   tibble::column_to_rownames("otu")
795
796 #Idem for the two other matrixes
797 tax_mat <- tax_mat %>%
798   tibble::column_to_rownames("otu")
799
800 samples_df <- samples_df %>%
801   tibble::column_to_rownames("sample")
802
803 #Transform into matrixes otu and tax tables (sample table can be left as data frame)
804 otu_mat <- as.matrix(otu_mat)
805 tax_mat <- as.matrix(tax_mat)
806
807 #Transform to phyloseq objects
808 library(phyloseq)
809 OTU = otu_table(otu_mat, taxa_are_rows = TRUE)
810 TAX = tax_table(tax_mat)
811 samples = sample_data(samples_df)
812 colnames(TAX) = c("Kingdom", "Phylum", "Class", "Order",
813   "Family", "Genus", "Species")
814
815 phylo_me <- phyloseq(OTU, TAX, samples)
816 phylo_me
817
818 #run ANCOM-BC
819 Library(ANCOMBC)
820 res = ancombc(phyloseq = phylo_me,
821   formula = "Study + ARDS",
822   p_adj_method = "fdr", lib_cut = 1000,
823   group = "ARDS", struc_zero = TRUE, neg_lb = TRUE, tol = 1e-5,
824   max_iter = 100, conserve = TRUE, alpha = 0.20, global = TRUE
825 )
826
827 res = out$res
828
829 Association plots and ROC curve plot
830 Association plots and ROC curve plot were plotted using scripts from siamcat package 49 We applied
831 specific parameter for filtering as described below. We summarized the significant findings from the 2
832 previous analyses (MWU test blocked and ANCOM adjusted, those with a FDR corrected p-value <
833 0.20) that were used to plot the association plot and the ROC curve plot. Thus, the siamcat package was
834 only used for plotting purpose, not for the detection of the microbiome signatures. The reason for this is
835 that we were not able to adjust for confounding factors (each, study) using siamcat. That it is why we
836 performed first specific tests with adjustment on the confounding factor. Thus, the plot of the differential
837 relative abundance in ARDS, HAP, and prolonged mechanical ventilation combined the results of the
838 MWU blocked test and the ANCOM adjusted.
839
840 #filter
841 sc.obj <- filter.features(sc.obj,

```

```

842         filter.method = 'abundance',
843         cutoff = 0.001)
844 sc.obj <- filter.features(sc.obj, cutoff=0.05,
845         filter.method='prevalence',
846         feature.type = 'filtered')
847 #plot associations
848 sc.obj <- check.associations(
849     sc.obj,
850     sort.by = 'fc',
851     alpha = 0.20, # that is to include all the discovered makers from MWU and ANCOM
852     mult.corr = "fdr",
853     detect.lim = 10 ^-6,
854     plot.type = "quantile.box",
855     panels = c("fc", "auroc"),
856     fn.plot = './plot_differiating_taxa.pdf', color.scheme = body_cols)
857 associations(sc.obj)
858 write.table(associations(sc.obj), "./table_list_taxa.txt", sep="\t", color.scheme = body_cols)
859 #test the model - model building – plot the ROC curve – interpretation plot
860 sc.obj <- normalize.features(
861     sc.obj,
862     norm.method = "log.unit",
863     norm.param = list(
864         log.n0 = 1e-06,
865         n.p = 2,
866         norm.margin = 1
867     )
868 )
869 sc.obj <- create.data.split(
870     sc.obj,
871     num.folds = 10,
872     num.resample = 10
873 )
874
875 sc.obj <- train.model(
876     sc.obj,
877     method = "lasso"
878 )
879 model_type(sc.obj)
880 models <- models(sc.obj)
881 models[[1]]
882 sc.obj <- make.predictions(sc.obj)
883 pred_matrix <- pred_matrix(sc.obj)
884 sc.obj <- evaluate.predictions(sc.obj)
885 model.evaluation.plot(sc.obj, fn.plot = './base_plot.pdf')
886 model.interpretation.plot(
887     sc.obj,
888     fn.plot = './ROC_curve.pdf',
889     consens.thres = 0.5,
890     limits = c(-3, 3),
891     heatmap.type = 'zscore',
892 )
893
894 Fast and frungal trees procedure

```

895 To aid clinical decision-making, we fitted a Fast and Frugal Tree (FFT) to predict risk groups accurately
896 ⁴⁹ FFTs are simpler versions of decision trees and have been shown to perform competitively with
897 random forests and to facilitate biologic interrogation by decreasing the number of key features. The
898 selected top features associated with ARDS or HAP, found using a Mann-Whitney U test blocked for
899 ‘study’ and ANCOM with adjusting for ‘study’ and with a p-value with a False Discovery Rate (FDR
900 corrected p-value) of 0.20, were then introduced in the FFT procedure. The FFT procedure only included
901 the selected features for each specific signature, not all the ASV table. The R script we used is described
902 below:

```
903 devtools::install_github("ndphillips/FFTrees", build_vignettes = TRUE)
904 library(FFTrees)
905 set.seed(1234)
906 ind <- sample(2, nrow(model), replace = T, prob = c(0.8, 0.2))
907 train <- model[ind==1,]
908 test <- model[ind==2,]
909 # Tree Model
910 library(FFTrees)
911 tree <- FFTrees(formula = ARDS ~ .,
912                 data = train,
913                 data.test = test,
914                 main = "ARDS Decisions",
915                 decision.labels = c("no_ARDS", "ARDS"))
916 tree
917 inwords(tree)
918 summary(tree)
919 names(tree)
920
921 tree$criterion_name
922 tree$cue_names
923 tree$formula
924 tree$data
925 tree$params
926 tree$competition
927 tree$cues
928
929 # Plot
930 pdf('FFT.pdf,width=7,height=7);
931 plot(tree)
932 dev.off()
933
```

934 *Validation of the microbiome signatures*

935 To validate the pertinence of the microbiome signatures, we used the risk index procedure that we
936 developed previously ⁴⁹ Briefly, to assess the value and the interest of a microbiome signature, we built
937 the risk index, calculated using the sum of relative abundances of the taxa that were significantly
938 associated with the occurrence of the outcome minus the sum of the relative abundances of the taxa that
939 were associated the absence of occurrence of the outcome. This risk index is therefore able to summarize
940 all the significant microbiome signatures linked the outcome in a given sample into a single number.

941 # Derives an risk index: bad bugs minus good bugs

```

942 # for y == TRUE
943 # x is taxon/OTU/feature table
944 # y is TRUE/FALSE for disease or other phenotype
945 # alpha determines FDR threshold for inclusion of bugs
946 # return value includes risk.index.f, a function that takes a matrix with the same
947 # named columns as x and returns the risk index for each row.
948 # diff.tests can be included as a prior result to avoid recomputation
949 # this should be output from differentiation.test(x,y) as described previously

950 "get.risk.index" <- function(x, y,
951     alpha=0.20,
952     threshold.method=c('acc','spec')[1],
953     threshold.spec=.80,
954     transform.type=c('none','asin-sqrt','sqrt')[1],
955     threshold=NULL,
956     eps=NULL,
957     parametric=FALSE, verbose=FALSE,
958     diff.tests=NULL
959 ){
960   if(any(is.na(y))) stop('y must not contain NA values')
961   if(any(is.na(x))) stop('x must not contain NA values')
962   require('ROCR')

963
964   x <- data.transform(x,transform.type)
965   if(is.null(diff.tests)){
966     diff.tests <- differentiation.test(x, y, alpha=alpha, parametric=parametric)
967   }
968
969   # if there are a range of alpha values, build multiple lists of hits
970   hit.ix <- which(diff.tests$qvalues <= alpha)
971
972   if(length(hit.ix) == 0){
973     warning('No hits found, predicting risk of 0 for all patients\n')
974     good.bugs <- NULL
975     bad.bugs <- NULL
976     sum.good.bugs <- NA
977     sum.bad.bugs <- NA
978     risk.index <- rep(0,nrow(x))
979     risk.index.f <- function(x) {return (rep(0,nrow(x)))}
980     threshold <- NA
981   } else {
982     if (verbose) {
983       cat("There were",length(hit.ix),"taxa significant at FDR of",alpha,"\n")
984     }
985
986     # get rid of zeros (if eps > 0)
987     if(!is.null(eps)) x[x==0] <- eps
988
989     # identify good/bad bugs
990     good.bugs <- diff.tests$classwise.means[,1] > diff.tests$classwise.means[,2] &
991     diff.tests$qvalues < alpha
992     bad.bugs <- diff.tests$classwise.means[,1] < diff.tests$classwise.means[,2] &
993     diff.tests$qvalues < alpha
994
995     sum.good.bugs <- numeric(nrow(x))

```

```

996     sum.bad.bugs <- numeric(nrow(x))
997     if(sum(good.bugs) > 0) sum.good.bugs <- rowSums(x[,good.bugs,drop=F])
998     if(sum(bad.bugs) > 0) sum.bad.bugs <- rowSums(x[,bad.bugs,drop=F])
999
1000    # risk index is simply additive bad bugs vs good bugs
1001    risk.index <- sum.bad.bugs - sum.good.bugs
1002
1003    # risk.index.f
1004    "risk.index.f" <- function(x){
1005        sum.good.bugs.i <- numeric(nrow(x))
1006        sum.bad.bugs.i <- numeric(nrow(x))
1007        if(sum(good.bugs) > 0) sum.good.bugs.i <- rowSums(x[,good.bugs,drop=F])
1008        if(sum(bad.bugs) > 0) sum.bad.bugs.i <- rowSums(x[,bad.bugs,drop=F])
1009        return(sum.bad.bugs.i - sum.good.bugs.i)
1010    }
1011
1012    # choose threshold based on requested threshold method
1013    if(is.null(threshold)){
1014        if(threshold.method=='acc'){
1015            # get threshold that is most accurate for this risk index
1016            pred <- prediction(risk.index, y)
1017            perf <- performance(pred, 'acc')
1018            threshold.ix <- which.max(perf@y.values[[1]])
1019            threshold <- perf@x.values[[1]][threshold.ix]
1020        } else if(threshold.method == 'spec'){
1021            # get threshold that is most accurate for this risk index
1022            pred <- prediction(risk.index, y)
1023            perf <- performance(pred, 'sens', 'spec')
1024            threshold.ix <- max(which(perf@x.values[[1]] >= threshold.spec))
1025            threshold <- perf@alpha.values[[1]][threshold.ix]
1026        } else {
1027            stop(paste('Unknown threshold method:', threshold.method))
1028        }
1029    }
1030 }
1031
1032 res <- list(
1033     risk.index=risk.index,
1034     risk.index.f=risk.index.f,
1035     diff.tests=diff.tests,
1036     good.bugs=good.bugs,
1037     bad.bugs=bad.bugs,
1038     hit.ix=hit.ix,
1039     alpha=alpha,
1040     threshold=threshold
1041 )
1042 class(res) <- "risk.index"
1043 return(res)
1044 }
1045
1046
1047 Receiving-operating characteristic (ROC) curve analysis
1048 # We applied tenfold jack-knifing; the ten ROC curves are in blue and the mean ROC curve is in black
1049 # runs jackknife ROC and plots a smoothed ROC curve; returns AUC
1050 # bootstrap.fraction = -1 means leave-one-out

```

```

1051 # outcome must be T/F
1052 "bootstrapped.ROC" <- function(predictors, outcome, nreps=10,
1053                               bootstrap.fraction=.9,
1054                               filename='ROC-signature.pdf', do.plot=TRUE,
1055                               title.text='signature', include.xy=TRUE){
1056   require('ROCR')
1057   if(is.null(dim(predictors))) predictors <- matrix(predictors,ncol=1)
1058   N <- nrow(predictors)
1059   if(bootstrap.fraction == -1) {
1060     folds <- sapply(1:N,function(ixx) (1:N)[-ixx])
1061   } else {
1062     bootstrap.n <- max(min(round(bootstrap.fraction * N), N-1),2)
1063     folds <- replicate(nreps,sample(N,size=bootstrap.n))
1064   }
1065   tprs <- NULL
1066   fprs <- NULL
1067   aucs <- NULL
1068
1069   for(fold in 1:nreps){
1070     fold.ix <- folds[fold]
1071     outcome.i <- outcome[fold.ix]
1072     predictors.i <- predictors[fold.ix,,drop=F]
1073     res <- logistic.ROC(predictors.i, outcome.i)
1074
1075     tprs <- cbind(tprs, res$tprs)
1076     fprs <- cbind(fprs, res$fprs)
1077     aucs <- c(aucs, res$auc)
1078   }
1079
1080
1081   require('flux')
1082   fprs.mean=rowMeans(fprs)
1083   tprs.mean=rowMeans(tprs)
1084   auc.mean <- auc(fprs.mean,tprs.mean)
1085
1086   res <- list(fprs.mean=fprs.mean, tprs.mean=tprs.mean,
1087             fprs=fprs, tprs=tprs,
1088             auc.mean=auc.mean, aucs=aucs,
1089             nreps=nreps,
1090             folds=folds)
1091   if(do.plot) plot.jackknifed.ROC(res, filename=filename, title.text=title.text,
1092 include.xy=include.xy)
1093
1094   invisible(res)
1095 }
1096
1097 Kaplan-Meier curves in the validation cohort
1098 To plot the Kaplan-Meier curves in the validation cohort, we adapted the cut-offs of the signatures,
1099 based on the ratio mean relative abundance of the signature in the IBIS cohort / mean relative abundance
1100 of the signature in the metanalysis. The following code was applied:
1101 ggsurvplot(
1102   fit, # survfit object with calculated statistics.
1103   data = ventil_prediction, # data used to fit survival curves.
1104   risk.table = TRUE, # show risk table.
1105   pval = TRUE, # show p-value of log-rank test.

```

```

1106 conf.int = TRUE,      # show confidence intervals for
1107 # point estimates of survival curves.
1108 xlim = c(0,50),      # present narrower X axis, but not affect
1109 # survival estimates.
1110 break.time.by = 10,  # break X axis in time intervals by 10.
1111 ggtheme = theme_minimal(), # customize plot and risk table with a theme.
1112 risk.table.y.text.col = T, # colour risk table text annotations.
1113 risk.table.y.text = FALSE # show bars instead of names in text annotations
1114 # in legend of risk table
1115 )

```

1116 *Classification Decision Tree*

1117 In complement to FFT, we also test Classification Decision Tree, as the latter may improve the accuracy and interpretability of the models. We used the following scripts :

```

1120
1121 set.seed(1234)
1122 ind <- sample(2, nrow(mydata), replace = T, prob = c(0.8, 0.2))
1123 train <- mydata[ind == 1,]
1124 test <- mydata[ind == 2,]
1125 #Tree Classification
1126 tree <- rpart(VAP ~., data = train)
1127 rpart.plot(tree)
1128
1129 printcp(tree)
1130 plotcp(tree)
1131 tree <- rpart(VAP ~., data = train,cp= ) #choose cp based on tree
1132 p <- predict(tree, train, type = 'class')
1133 #confusionMatrix(p, train$aids, positive="yes")
1134
1135 p1 <- predict(tree, test, type = 'prob')
1136 p1 <- p1[,2]
1137 r <- multiclass.roc(test$VAP, p1, percent = TRUE)
1138 roc <- r[['rocs']]
1139 r1 <- roc[[1]]
1140 plot.roc(r1,
1141         print.auc=TRUE,
1142         auc.polygon=TRUE,
1143         grid=c(0.1, 0.2),
1144         grid.col=c("green", "red"),
1145         max.auc.polygon=TRUE,
1146         auc.polygon.col="lightblue",
1147         print.thres=TRUE,
1148         main= 'ROC Curve')
1149

```

1150 **Supplemental results**

1151 The total output for the sequencing of samples, after quality trimming, removal of duplicates, human
1152 genome sequences and chimeras a total of 872,155 features remained for analysis (total frequency:
1153 80,302,231). The sequence count per sample ranged from 1 to 715,298 with a median of 16,993 (1st
1154 quartile: 8,478 and 3rd quartile: 32,264) and a mean of 27,092.

1155

1156

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1287 **FIGURES LEGEND**

1288

1289 **Figure 1. Respiratory microbiome alterations along critically ill patient airways**

1290 **A.** Beta diversity comparisons of the respiratory microbiomes of the all samples (including BAL, ETA
1291 and OS) collected from ICU patients and healthy controls. Analyses were performed on the 16S rRNA
1292 gene. Principal-coordinate analysis of Bray-Curtis distances. The proportion of variance explained by
1293 each principal-coordinate axis is denoted in the corresponding axis labels. **B.** Alpha diversity indices in
1294 all the samples (including BAL, ETA and OS) collected from ICU patients and healthy controls were
1295 measured using the Shannon index (left panel) or the number of observed genera (right panel). **C.**
1296 Relative abundance of the healthy respiratory microbiome core genus in respiratory samples from
1297 healthy controls and critically ill patients. We included samples collected at BAL, ETA, and OS. **D.**
1298 Network correlation plot of the core respiratory microbiome in the respiratory samples collected in
1299 critically ill patients. Negative correlations are represented in blue, and positive ones are in red. The cut-
1300 off for prevalence was the presence of a genus in at least 50% of the cohort. We included samples
1301 collected at BAL, ETA, and OS. **E.** Alpha diversity indices in BAL, ETA, and OS collected from ICU
1302 patients were measured using the Shannon index (left panel) or the number of observed genera (right
1303 panel). **F.** Network correlation plots of the respiratory microbiome in BAL, ETA, and OS collected in
1304 critically ill patients. Negative correlations are represented in blue, and positive ones are in red. The cut-
1305 off for prevalence was the presence of a genus in at least 50% of the cohort. **G.** Sum of the genera of the
1306 core healthy microbiome (relative abundance) in BAL, ETA, and OS collected in critically ill patients.
1307 **H.** Respiratory microbiome core of critically ill patients. UpSet plot of the core respiratory microbiome
1308 in the 2177 samples collected in intensive care unit patients, including BAL, ETA, and OS. The cut-off
1309 for prevalence was the presence of the genus in at least 40% of the cohort of patients in each sampling
1310 site. Statistical significance was assessed by the Bray-Curtis distance (PERMANOVA) and two-sided
1311 Mann-Whitney test for the principal coordinates (**A**) and Kruskal-Wallis with Dunn's tests (**B,E,G**). *P
1312 ≤ 0.05 , **P ≤ 0.01 , ***p <0.001 .

1313

1314 **Figure 2. Respiratory microbiome signatures of ARDS in endotracheal aspirates**
1315 **A.** Beta diversity comparisons of the respiratory microbiomes of the ETA collected from ICU patients
1316 with or without ARDS. Analyses were performed on 16S rRNA gene. Principal-coordinate analysis of
1317 Bray-Curtis distances. The proportion of variance explained by each principal-coordinate axis is denoted
1318 in the corresponding axis labels. **B-C.** Alpha diversity indices in ETA samples collected from critically
1319 ill patients with or without ARDS were measured using **(B)** the Shannon index and **(C)** the number of
1320 observed genera. **D.** Relative abundance of the main phyla in ETA samples from critically ill patients
1321 with or without ARDS. **E-F.** Network correlation plots of the core respiratory microbiome in the ETA
1322 samples collected in critically ill patients **(E)** without or **(F)** with ARDS. Negative correlations are
1323 represented in blue, and positive ones in red. The cut-off for prevalence was the presence of a genus in
1324 at least 50% of the cohort. **G.** Top features at genus level with different relative abundance in ETA of
1325 critically ill patients with ARDS and no ARDS. The function computes for each genus the significance
1326 using a non-parametric Wilcoxon test and different effect sizes for the association (significance for an
1327 FDR-corrected p-value, AUC, and fold change). **H.** Fast-and-frugal tree-based staging scheme to predict
1328 ARDS in ICU patients. **I.** ROC of ARDS classification applying a ten-repeated 10-fold cross-validation
1329 scheme. The model evaluation displays the cross-validation error as a receiver operating characteristic
1330 (ROC) curve, with a 95% confidence interval shaded in grey. **J.** Kaplan-Meier estimates the time-to-
1331 successful extubation in patients with ARDS vs no ARDS microbiome signature (mortality treated by
1332 censoring). Statistical significance was assessed by the Bray-Curtis distance (PERMANOVA) and two-
1333 sided Mann-Whitney test for the principal coordinates **(A)**, and Kruskal-Wallis with Dunn's tests **(B,**
1334 **C)**, and Cox proportional-hazards model **(J)**. *P ≤ 0.05, **P ≤ 0.01, ***p<0.001.
1335

1336 **Figure 3. Respiratory microbiome signatures of HAP in endotracheal aspirates**
1337 **A.** Beta diversity comparisons of the respiratory microbiomes of the ETA collected from critically ill
1338 patients with or without HAP. Analyses were performed on 16S rRNA gene. Principal-coordinate
1339 analysis of Bray-Curtis distances. The proportion of variance explained by each principal-coordinate
1340 axis is denoted in the corresponding axis labels. **B-C.** Alpha diversity indices in ETA samples collected
1341 from critically ill patients with or without HAP were measured using **(B)** the Shannon index and **(C)** the
1342 number of observed genera. **D.** Relative abundance of the main phyla in ETA samples from critically ill
1343 patients with or without HAP. **E-F.** Network correlation plots of the core respiratory microbiome in the
1344 ETA samples collected in critically ill patients **(E)** without or **(F)** with HAP. Negative correlations are
1345 represented in blue, and positive correlations are represented in red. The cut-off for prevalence was the
1346 presence of a genus in at least 50% of the cohort. **G.** Top features at genus level with different relative
1347 abundance in ETA of critically ill patients with HAP and no HAP. The function computes for each genus
1348 the significance using a non-parametric Wilcoxon test and different effect sizes for the association
1349 (significance for an FDR corrected p-value, AUC, and fold change). **H.** Fast-and-frugal tree-based
1350 staging scheme to predict HAP. **I.** ROC of the HAP classification applying a ten-repeated 10-fold cross-
1351 validation scheme. The model evaluation displays the cross-validation error as a receiver operating
1352 characteristic (ROC) curve, with a 95% confidence interval shaded in grey. **J.** Kaplan-Meier estimates
1353 the time-to-successful extubation in patients with HAP vs no HAP microbiome signature (mortality
1354 treated by censoring). Statistical significance was assessed by the Bray-Curtis distance (PERMANOVA)
1355 and two-sided Mann-Whitney test for the principal coordinates **(A)**, and Kruskal-Wallis with Dunn's
1356 tests **(B, C)**, and Cox proportional-hazards model **(J)**. *P ≤ 0.05, **P ≤ 0.01, ***p<0.001.
1357

1358 **Figure 4. Respiratory microbiome signatures of successful extubation in endotracheal aspirates**
1359 **A.** Top features at genus level with different relative abundance in ETA of critically ill patients with
1360 early vs. late successful extubation. The function computes for each genus the significance using a non-
1361 parametric Wilcoxon test and different effect sizes for the association (significance for an FDR-
1362 corrected p-value, AUC, and fold change). Early extubation: <4 days of mechanical ventilation; late
1363 extubation: >10 days of mechanical ventilation (mortality treated by censoring). **B.** Fast-and-frugal tree-
1364 based staging scheme for time-to-successful extubation classification. **C.** ROC of the late successful
1365 extubation classification applying a ten-repeated 10-fold cross-validation scheme. The model evaluation
1366 displays the cross-validation error as a receiver operating characteristic (ROC) curve, with a 95%
1367 confidence interval shaded in gray.

1368 **Figure 5. External validation of respiratory microbiome signatures in an independent prospective**
1369 **cohort**

1370 **A-C.** Beta diversity comparisons of the respiratory microbiomes of the ETA collected from brain-
1371 injured patients (A) with or without ARDS, (B) with or without HAP or (C) with early, media or late
1372 successful extubation classification. Principal-coordinate analysis of Bray-Curtis distances. The
1373 proportion of variance explained by each principal-coordinate axis is denoted in the corresponding axis
1374 labels. **D-F.** Shannon index indices in ETA samples collected from critically ill patients (D) with or
1375 without ARDS, (E) with or without HAP, and (F) with early, media or late successful extubation. **G-I.**
1376 ROC of Fast-and-frugal tree for (G) ARDS, (H) HAP, and (I) early successful extubation applying a
1377 tenfold jack-knifing; the ten ROC curves are in blue and the mean ROC curve is in black. **J-L.** Kaplan-
1378 Meier estimates the time-to-successful extubation in patients stratified by microbiome scores for (J)
1379 ARDS, (K) HAP and (L) early successful extubation. Statistical significance was assessed by the Bray-
1380 Curtis distance (PERMANOVA) and two-sided Mann-Whitney test for the principal coordinates (**A-C**),
1381 and Kruskal-Wallis with Dunn's tests (**D-F**), and Cox proportional-hazards model (**J-L**). *P ≤ 0.05, **P
1382 ≤ 0.01, ***p<0.001.

1383

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1385 **Author contributions:** E.M., GDK, RPD and A.R. selected studies, performed data analyses,
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1397 **Data availability.** Correspondence and requests should be addressed to A.R. (antoine.roquilly@univ-
1398 nantes.fr). 16S rRNA sequence dataset of the validation cohort has been deposited in a secure file by
1399 CHU Nantes (HAP2 data hub) and a public repository
1400 (<https://submit.ncbi.nlm.nih.gov/about/sra/#step1>). The data for the studies included in the metanalysis
1401 can be found as figshare: [https://figshare.com/s/](https://figshare.com/s/46c402c6465c8a289c18)
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1407 PRJNA595346, SRP112361, PRJNA553560, PRJNA553560, SRP076183, PRJNA339755). The
1408 accession for each study is reported in supplemental tables 1, 2 and 3. Access to metadata containing
1409 potentially identifying patient information requires an approved research ethics protocol and may
1410 require approval from CHU Nantes as the steward of patient information for all study participants; a
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