Biological effects of pulmonary, blood and gut microbiome alterations in patients with acute respiratory distress syndrome

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To the Editor:

Alterations in pulmonary and gut microbiome are associated with an impairment in clinical outcomes in critically ill patients, including those with acute respiratory distress syndrome (ARDS) [1–6]. Although experimental studies have suggested gut-to-lung bacterial translocation [6], there is limited knowledge of simultaneous microbiome changes across different body compartments in patients with ARDS. Additionally, the biological consequences of these alterations at different body sites have not been sufficiently explored. Therefore, the aim of this study is to study the gut, lung and blood microbiome composition in patients with ARDS, and analyse its relationship to the systemic biological response.

This observational study aimed to describe the differences in microbiome composition across various body sites and the biological response in terms of inflammation, epithelial and endothelial dysfunction, and coagulopathy in patients with ARDS. We included patients undergoing mechanical ventilation who met the Berlin definition criteria [7] at the Hospital Clinic of Barcelona. The exclusion criteria were: 1) mechanical ventilation for more than 72 h before inclusion; 2) arterial oxygen tension (P_{aO_2})/inspiratory oxygen fraction (F_{IO_2}) >300 within 24 h of inclusion; 3) expected decisions to withdraw life-sustaining treatment within 24 h; 4) refusal to participate in the study; and 5) previously known interstitial lung disease. Endotracheal aspirate (ETA), blood, rectal swab samples and plasma were collected upon ARDS diagnosis.

The DNA extraction from the ETA, blood and rectal swab samples was accomplished using the Norgen DNA sputum kit, Norgen DNA Blood kit and Norgen DNA stool kit (all Norgen Corp., ON, Canada), respectively, following the instructions provided by the manufacturer. Extracted DNA was quantified with the Qubit system (Thermo Fisher Scientific, MA, USA). The DNA library preparation was based on the hypervariable regions V3–V4 of bacterial 16S RNA gene using the QIASeq Region Panel 16S (QIAGen, Hilden, Germany). They were created and purified by Agencourt AMPure XP Beads and short fragments (non-target products) were removed using the Ampure beads kit (Agencourt Bioscience, Waltham, MA, USA). We followed the amplification programme previously published [8]. The prepared DNA libraries were then assessed for quality and size using a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) with a DNA 7500 chip. The resulting pool of DNA libraries was sequenced using the Illumina MiSeq Sequencing system (Illumina, San Diego, CA, USA) at the IDIBAPS Genomic Core facilities. The amplicon sequence variants obtained in each sample were delivered in fastq format files for subsequent bioinformatic analyses.

Operational taxonomic units (OTUs) were obtained using the Silva database, integrated into the Qiime2 tools. Chimeric reads were identified using a non-chimeric 16S rRNA database from Qiime2, achieving a 99% similarity rate [9]. The OTUs underwent clustering into distinct groups using Bioconductor packages version 3.11. Subsequently, total bacterial reads were obtained for each sample; the relative abundance of the most frequent OTUs were calculated, and alpha and beta diversity indices were estimated. We performed negative controls sampling and DNA extraction and quantification of phosphate-buffered saline samples. However, none of the controls were sequenced because they exhibited DNA amounts below the sensitivity of the Qubit system.

Plasma levels of soluble receptor advanced glycation end-products, interferon-γ, interleukin (IL)-6, IL-8, IL-10, IL-17, IL 1-β, tumour necrosis factor-α, vascular endothelial growth factor, factor XI, protein C,

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There is an overlap between the respiratory, blood and gut microbiomes in patients with acute respiratory distress syndrome. Specific taxa in the lungs and blood are associated with an inflammatory response. https://bit.ly/3TdkHd7

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protein S, von Willebrand factor and plasminogen activator inhibitor were analysed with Luminex technology. ELISA kits were used for tissue factor, soluble receptor tumour necrosis factor α, surfactant D protein, angiopoietin and angiotensin-II.

Data are reported as frequencies and percentages for categorical variables and as medians and interquartile ranges for continuous variables. Categorical variables were compared using the Fisher's exact test. Continuous variables were compared using the Mann–Whitney U-test. Spearman correlation analysis was conducted to investigate the association between systemic cytokines and OTUs. The level of significance was set at 0.05 (two-tailed). All analyses were performed using GraphPad Prism version 10.1.2 (GraphPad Software, San Diego, CA, USA).

From June 2019 to March 2020, we obtained 44 samples (16 blood samples, 18 ETA samples and 10 rectal swabs) from 20 included patients. The median age was 65 (54–68) years and 15 (75%) patients were male. The Acute Physiology and Chronic Health Evaluation score at ICU admission was 21 (16.7–26.2). The time from hospital admission to ICU admission was 1 (0–3) days, and the time from ICU admission to ARDS diagnosis was 2 (1–3) days. The most common cause of ARDS was pneumonia (70%). Three (15%) and four (20%) patients had positive lower-respiratory and blood cultures, respectively. The median P_{aO_2}/F_{IO_2} was 163 (82–185), the ventilatory ratio was 2.26 (1.96–2.5), and the static respiratory system compliance was 32 (25–44) $mL \cdot cmH_2O^{-1}$. 17 (85%) patients were ventilated in volume-controlled mode. The median tidal volume was 6.8 (6.2–7.9) $mL \cdot kg^{-1}$ predicted body weight, the positive end-expiratory pressure was 10 (8–12) cmH_2O, and the positive end-inspiratory and driving pressures were 24 (20–27) and 13 (11–14) cmH_2O, respectively. Six (30%) patients were placed in prone position and received neuromuscular blocking agents, and three patients were treated with extracorporeal membrane oxygenation. 13 patients (65%) received systemic corticosteroids. The median number of reads in ETA samples was 6590 (131–23 459), corresponding to 17 (11–24) OTUs. In blood samples, the median was 31 659 (23 311–41 054), corresponding to 21 (18–23) OTUs, while in rectal swabs, it was 72 174 (52 290–102 412), corresponding to 63 (56–73) OTUs.

The relative abundance of the 10 most abundant OTUs in ETA, blood and rectal swab samples are displayed in figure 1a. The most abundant OTUs were: *Bacilli* class from the phylum *Firmicutes* in ETA (relative abundance 23.5%); phylum *Bacteroidetes* (relative abundance 38.1%) in blood; and the *Gammaproteobacteria* class from the phylum *Proteobacteria* (relative abundance 15.4%) in gut. The *Bacteroidales* order, typical gut commensal bacteria, was detected in all faecal samples and in most blood samples (94% detection) and respiratory samples (94% detection). It was the third and the sixth most frequent OTU found in ETA and blood samples (relative abundance 8.9% and 2.9%, respectively). The *Enterobacteriaceae* family was present in 22%, 44% and 80% of respiratory, blood and rectal samples, respectively.

α-diversity was higher in the gut microbiome compared to other body sites (gut Shannon index 2.8 (2.5–2.9)). However, the blood and respiratory microbiomes presented with similar Shannon indices (1.9 (1.6–2.3) and 1.9 (1.4–2.4), respectively) (figure 1b). In figure 1c, we depict the β-diversity between body sites. The blood and respiratory microbiomes shared 23% of OTUs. Moreover, 17% of OTUs found in the gut were also present in the respiratory microbiome.

The relative abundance of the *Bacteroidetes* phylum and the *Bacteroidales* order in lower respiratory samples exhibited positive correlations with inflammation, pulmonary epithelial dysfunction, endothelial dysfunction and coagulation disorders (figure 1d). Additionally, the presence of the *Proteobacteria* phylum in the blood showed a negative association with inflammatory cytokines (figure 1e).

The major findings of this study can be summarised as follows: first, we observed an overlap of approximately 20% between the respiratory, blood and gut microbiomes. Second, the presence of the *Bacteroidales* order in the lungs was associated with higher levels of inflammation, epithelial and endothelial pulmonary dysfunction, and coagulation disorders. Third, despite the low rate of positive blood cultures (15%), we observed bacterial DNA leakage in all blood samples. Finally, the relative abundance of the *Proteobacteria* phylum in blood was related to lower levels of inflammation.

To study the gut-to-lung translocation, an experimental study using a rodent model of abdominal sepsis-induced ARDS identified *Bacteroidales* as the primary gut-derived OTU that translocated into the lungs [6]. That study also explored whether *Bacteroidales* translocation occurred in patients with ARDS and in healthy subjects [6]. This enteric OTU was found in 33% of bronchoalveolar lavage fluid samples from patients with ARDS, compared to 3% in bronchoalveolar lavage samples from healthy subjects, and

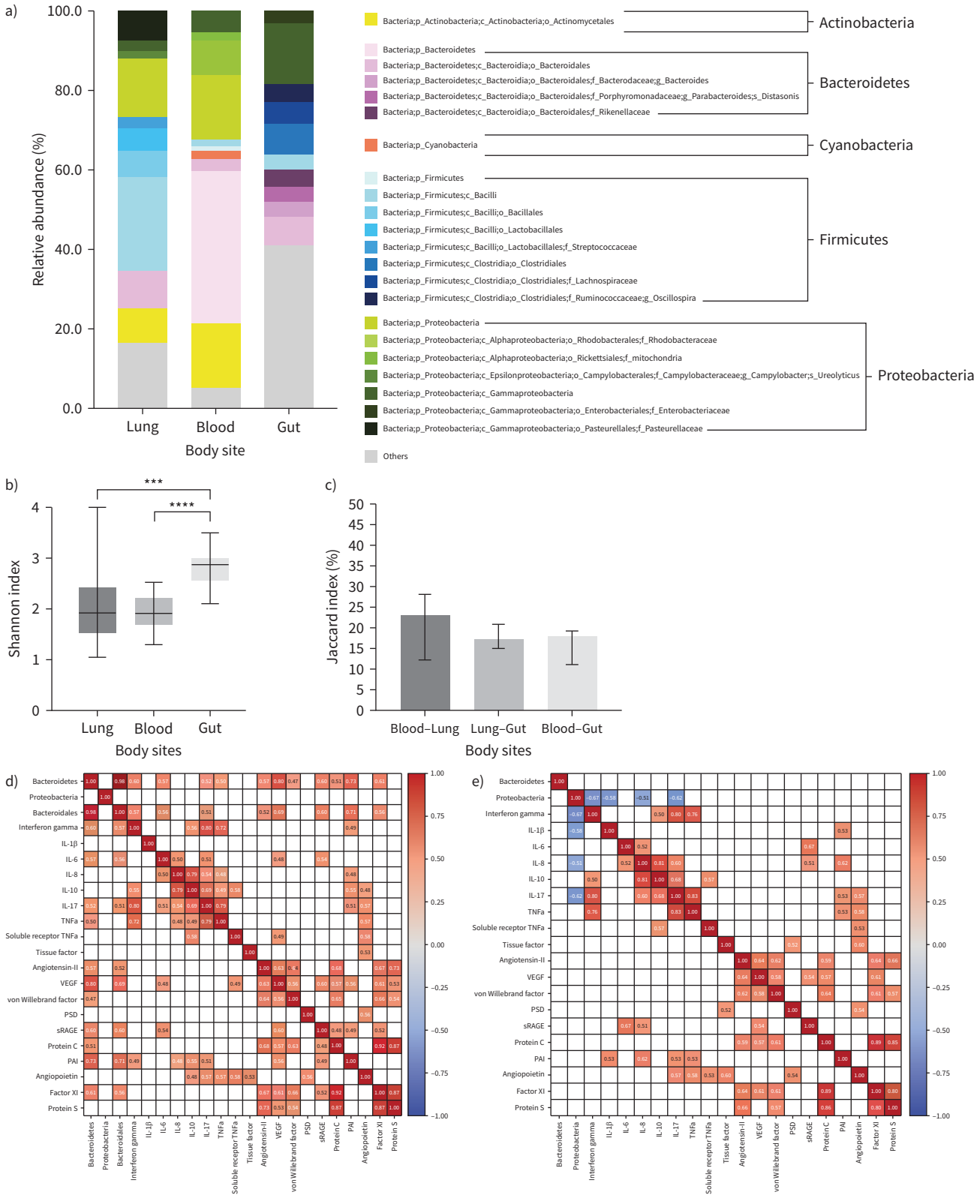

FIGURE 1 a) Relative abundance of bacteria in respiratory, blood and faecal samples. The 10 most abundant operational taxonomic units (OTUs) are displayed. OTUs that did not reach the phylum level were removed. b) Shannon indexes of gut, lung and blood microbiome. ***: p<0.001; ****: p<0.0001. c) Jaccard similarity index of paired body compartments. d) Correlation heatmap between *Bacteroidetes* and *Proteobacteria* phylum, *Bacteroidales* order found in endotracheal aspirates and plasma cytokines. Coloured boxes are statistically significant, showing the correlation coefficient between cytokines and the relative abundance of each OTU. e) Correlation heatmap between *Bacteroidetes* and *Proteobacteria* phylum,

Bacteroidales order found blood and plasma cytokines. Coloured boxes are statistically significant, showing the correlation coefficient between cytokines and the relative abundance of each OTU. IL: interleukin; PAI: plasminogen activator inhibitor; PSD: surfactant D protein; sRAGE: soluble receptor for glycation end-products; TNF: tumour necrosis factor; VEGF: vascular endothelial growth factor.

94% in ETA samples in our study. They also assessed the similarity between the faecal and pulmonary microbial communities in their experimental model. The Jaccard similarity index between the respiratory and faecal samples was approximately 0.3, indicating that 30% of OTUs were shared between these two body compartments. In our study, however, the similarity between respiratory and faecal microbiomes was slightly lower, possibly due to the different ARDS aetiology in our cohort (mainly pneumonia-induced ARDS). Notably, we identified that the respiratory and blood microbiomes exhibited the highest degree of similarity.

Interestingly, the abundance of *Bacteroidetes* and *Bacteroidales* in the lungs were associated with a variety of systemic biological responses, including inflammation, endothelial dysfunction, pulmonary epithelial dysfunction and coagulation disorders. Other studies have reported similar results when analysing the association between specific bacterial clusters and the systemic inflammatory response in a general cohort of patients undergoing mechanical ventilation [2]. Establishing causality for such associations would be of great interest, as targeting the migration of gut bacteria could potentially be a treatable trait in patients with ARDS.

One crucial finding in our study is the identification of bacterial DNA in the blood, suggesting occult bacteraemia not detected by conventional blood cultures. The existence of a blood microbiome has been suggested in both healthy subjects and critically ill patients [10–12]. In near half of the blood samples we identified the presence of *Enterobacteriaceae*, a family that comprehends several pathogenic enteric species. One study on sepsis also found that *Enterobacteriaceae* species were present in the blood of patients, specifically in a greater proportion among those with the hyperinflammatory subphenotype [12]. Blood microbiome in healthy subjects has a predominance of *Proteobacteria* phylum bacteria of undetermined source [10, 11]. In contrast, our study in ARDS patients revealed *Bacteroidetes* as the principal phylum in the blood microbiome. Furthermore, we found that the relative abundance of the *Proteobacteria* phylum, the one observed in healthy subjects [10, 11], was associated with lesser systemic inflammatory response.

Our study has some limitations. The main limitation, due to its design as a pilot study, is that both the results and conclusions are constrained by a lack of statistical power. Therefore, new studies with larger sample sizes are needed to validate our findings. Second, our methodology does not allow us to establish cause–effect relationships. Third, due to the small sample size, our population does not cover the wider ARDS aetiology. Fourth, samples were obtained upon ARDS diagnosis at a single time point. Fifth, although samples were obtained under sterile conditions, we cannot fully rule out some degree of contamination. Sixth, bronchoalveolar lavage samples might have yielded different results compared to ETA samples.

In conclusion, we have described the microbiome profile of ARDS patients, identifying overlap between body compartments and demonstrating associations between specific taxa and cytokines of epithelial and endothelial dysfunction, coagulation and inflammation.

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