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Characterization of lung microbiota in pneumonia: a pilot study in ICU and non-ICU patients

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Abstract

Objective To identify potential differences in lung microbiota according to clinical severity, age, and gender in pneumonia patients compared to controls.

Design Pilot study.

Setting Single center (Azienda Ospedaliera Universitaria Dulbecco, Catanzaro, Italy).

Patients Thirty-three individuals—11 ICU patients requiring invasive mechanical ventilation, 11 non-ICU patients, and 11 cadaver controls without lung disease.

Interventions Bronchoalveolar lavage sample collection and analysis via microbiological cultures and metagenomic sequencing.

Measurements and main results Bacteroidota and Verrucomicrobiota phyla were more abundant in older (≥ 65 years) ICU and non-ICU patients versus controls. *Massilia timonae* showed a significantly lower relative abundance at the group level in cases compared to controls, potentially increasing infection susceptibility. Higher microbiota diversity was observed in older patients.

Conclusions Alterations in lung microbiota composition were observed in pneumonia patients, with differences that appeared more evident in older patients. Microbiota phenotyping may offer novel insights into pneumonia pathophysiology and pulmonary dysbiosis.

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Human microbiota has been defined as a complex community of microorganisms, including bacteria, fungi and viruses [1], playing vital roles in immune regulation, digestion, vitamin production, and disease prevention [1]. According to recent findings, lungs are inhabited by different microbial communities, remarkably altered in patients affected by chronic lung disease [2]. More specifically, lung microbiota in healthy volunteers is rich in *Bacteroidota* and *Bacillota* (mainly *Prevotella* and *Streptococcus*), while *Pseudomonadota* is prevalent in pulmonary disorders [2]. Indeed, in critically ill patients, dysbiosis is a common condition resulting from a severe microbial imbalance, which influences disease severity, hinders healing processes, and negatively affects clinical outcomes [2]. However, data on lung microbiota in patients affected by pneumonia remain limited, whereas more extensive evidence is currently available for chronic respiratory diseases, such as pulmonary fibrosis [1, 3]. Based on these findings, the present pilot study, conducted at the Azienda Ospedaliera Universitaria Dulbecco of Catanzaro (protocol approval n. 113, Aprile 20, 2023), enrolled adult patients affected by pneumonia [4] and excluded patients with length of stay in Intensive Care Unit (ICU) < 24 h (n. 2), pregnancy (n. 1), primary or secondary immunosuppression (n. 0) and recent receipt of probiotics (≤ 1 year) (n. 0). The primary aim was to identify potential differences in lung microbiota

according to clinical severity (i.e., 'ICU-patients,' requiring invasive mechanical ventilation (IMV) and 'non-ICU patients,' never exposed to IMV, as compared to 'controls' (i.e., cadavers (without lung disease), suddenly died due to trauma or cardiac arrest. In this group the biological sample was collected within 1 h after death) and, then, based on age (< or ≥ 65 years) and gender. Bronchoalveolar lavage (BAL) samples, collected according to current recommendations for bronchoscopy procedures [5, 6] and in response to clinical indications in cases (and within the first 48 h from the clinical suspicion) [7] were analyzed using microbiological cultures and metagenomic sequencing (as described in Table 1). Given the limited data available on lung microbiota in pneumonia, exploratory studies are needed to generate initial hypotheses. Due to the lack of data available on lung microbiota in pneumonia and the explorative nature of this study, a sample size of at least 10 subjects for each group was arbitrary considered adequate. Statistical analysis was performed using Microbiome Analyst and R-software; while, alpha and beta-diversity of lung microbiota were investigated as indicated in Fig. 1 [8, 9].

Overall, 33 individuals were enrolled, including 11 (33%) ICU patients, 11 (33%) non-ICU patients, and 11 (33%) healthy controls. The cohort comprised 21 males (63%) and 12 females (37%), with a mean age of 60.8 ± 20.3 years. Baseline characteristics are detailed

Table 1 Baseline characteristics

		ICU-patients N= 11 (33)	Non-ICU patients N= 11 (33)	Controls N= 11 (33)	p-value
Demographic characteristics					
	Age, years (mean)	70 \pm 23	63 \pm 20	47 \pm 15	< 0.001
	Females, n (%)	8 (72)	4 (36)	2 (18)	0.091
	BMI, kg/m ²	35 \pm 8	32 \pm 7	30 \pm 7	< 0.001
Comorbidities					
	Hypertension, n (%)	8 (72)	7 (63)	4 (36)	0.200
	Obesity, n (%)	1 (9)	1 (9)	4 (36)	0.090
	Diabetes, n (%)	4 (36)	6 (45)	3 (27)	0.150
	Hyperlipidemia, n (%)	2 (18)	2 (18)	2 (18)	0.320
	Coronary heart disease, n (%)	3 (27)	2 (18)	0 (0)	0.591
At enrolment					
	SOFA score	6 \pm 2	2 \pm 2	-	0.005

Table 1 (continued)

	Respiratory support, n (%)	IMV: 11 (100)	HFOT: 8 (73); COT: 3 (27)	-	-
	Vasoactive support, n (%)	9 (81)	0 (0)	-	< 0.001
	Antimicrobial therapies, n (%)	11 (100)	7 (64)	-	0.090
	Carbapenems	4 (36)	1 (9)	-	
	New BLIs/ β -lactam- β -lactamase inhibitor combinations	7 (64)	5 (45)	-	
	Anti-Gram-positive agents	3 (27)	3 (27)	-	
	pH	7.37 \pm 0.7	7.43 \pm 0.5	-	0.342
	PaO ₂ /FiO ₂	170 \pm 40	297 \pm 16	-	0.032
	pCO ₂ , mmHg	37 \pm 7	41 \pm 8	-	0.052
Pulmonary microbiota (phylas)*					
	Bacillota, relative abundance, %	< 65 yo (41.62 \pm 16.20); \geq 65 yo (34.21 \pm 7.36)	< 65 yo (48.45 \pm 13.23); \geq 65 yo (33.39 \pm 7.05)	< 65 yo (45.61 \pm 6.67); \geq 65 yo (38.58 \pm 16.05)	0.742
	Pseudomonadota, relative abundance, %	< 65 yo (28.54 \pm 20.48); \geq 65 yo (7.90 \pm 1.54)	< 65 yo (25.30 \pm 12.31); \geq 65 yo (23.98 \pm 8.89)	< 65 yo (15.18 \pm 3.05); \geq 65 yo (22.37 \pm 9.17)	0.474
	Bacteroidota, relative abundance, %	< 65 yo (6.44 \pm 5.50); \geq 65 yo (11.15 \pm 2.95)	< 65 yo (2.68 \pm 1.47); \geq 65 yo (13.45 \pm 3.51)	< 65 yo (8.49 \pm 3.38); \geq 65 yo (0.16 \pm 0.12)	0.050

Table 1 (continued)

	Actinomycetota, relative abundance, %	< 65 yo (3.15 \pm 1.67); \geq 65 yo (3.76 \pm 1.19)	< 65 yo (7.38 \pm 2.00); \geq 65 yo (5.26 \pm 0.96)	< 65 yo (6.37 \pm 1.38); \geq 65 yo (11.90 \pm 10.15)	0.493				
	Campylobacterota, relative abundance, %	< 65 yo (0.16 \pm 0.13); \geq 65 yo (0.25 \pm 0.10)	< 65 yo (0.05 \pm 0.04); \geq 65 yo (0.38 \pm 0.10)	< 65 yo (0.24 \pm 0.11); \geq 65 yo (0.00 \pm 0.00)	0.073				
	Fusobacteriota, relative abundance, %	< 65 yo (0.13 \pm 0.09); \geq 65 yo (0.04 \pm 0.02)	< 65 yo (0.05 \pm 0.02); \geq 65 yo (0.09 \pm 0.03)	< 65 yo (0.06 \pm 0.02); \geq 65 yo (0.02 \pm 0.02)	0.598				
	Verrucomicrobiota, relative abundance, %	< 65 yo (0.13 \pm 0.09); \geq 65 yo (0.43 \pm 0.13)	< 65 yo (0.07 \pm 0.07); \geq 65 yo (0.39 \pm 0.14)	< 65 yo (0.22 \pm 0.10); \geq 65 yo (0.00 \pm 0.00)	0.030				
	Unclassified bacteria, relative abundance %	< 65 yo (19.82 \pm 9.10); \geq 65 yo (42.25 \pm 10.56)	< 65 yo (16.03 \pm 6.10); \geq 65 yo (23.06 \pm 8.21)	< 65 yo (23.82 \pm 6.49); \geq 65 yo (26.97 \pm 7.11)	0.563				
		ICU-patients < 65 yo	ICU-patients \geq 65 yo	non-ICU patients < 65 yo	non-ICU patients \geq 65 yo	Controls < 65 yo	Controls \geq 65 yo	LDA score	p-value
Pulmonary microbiota (normalized relative abundance of species)*	<i>Akkermansia</i> sp.	1322	4330	699	3889	2239	0	3.3	0.029

Table 1 (continued)

<i>Bacteroides</i> sp.	12559	21437	2916	27666	15457	0	4.1	0.017
<i>Bifidobacterium</i> sp.	2402	4415	588	5083	3281	0	3.4	0.047
<i>Blautia</i> sp.	2599	4760	714	5670	3491	0	3.5	0.040
<i>Corynebacterium</i> sp.	7631	12064	1633	17256	11315	0	3.9	0.017
<i>Gemella haemolysans</i>	8515	22519	46790	1144	4970	57581	4.5	0.034
<i>Klebsiella</i> sp.	9229	13836	1550	19950	10933	26	4.0	0.033
<i>Massilia timonae</i>	473	10	14185	1393	8533	13966	3.9	0.026
<i>Massilia timonae</i> CIP: 105350_type	2664	105	92247	8623	45179	79284	4.7	0.008
<i>Mobiluncus</i> sp.	1105	1583	243	2229	1178	0	3.1	0.044
<i>Murdochiella</i> sp.	1247	2227	387	3314	1964	0	3.2	0.049
<i>Negativicoccus</i> sp.	1590	2105	218	3590	2211	0	3.3	0.044
<i>Paraclostridium</i> sp.	402	927	89	1170	1035	0	2.8	0.046
<i>Peptoniphilus</i> sp.	9032	16560	2247	23940	14250	0	4.1	0.034
<i>Phascolarctobacterium</i> sp.	1301	1974	365	3337	1552	0	3.2	0.039
<i>Porphyromonas</i> sp.	10750	15616	2073	20406	11903	0	4.0	0.045
<i>Romboutsia</i> sp.	934	2135	240	2637	1913	0	3.1	0.047

Table 1 (continued)

<i>Segatella</i> sp.	1293	1354	218	2264	1635	0	3.1	0.034
unclassified <i>Betaproteobacteria</i>	338	72	4756	705	3649	5653	3.5	0.007
unclassified <i>Bifidobacteriaceae</i>	403	940	138	1111	705	0	2.8	0.042
unclassified <i>Escherichia</i> and <i>Shigella</i>	4610	6179	707	9323	5506	0	3.7	0.044
unclassified <i>Oxalobacteraceae</i>	285	26	11453	1006	5260	7423	3.8	0.017
<i>Veillonella dispar</i>	855	6415	43468	502	83536	157110	4.9	0.012
	ICU-patients N= 11 (33)		Non-ICU patients N= 11 (33)		Controls N= 11 (33)		p-value	LDA score
<i>Massilia timonae</i>	164		6722		9892		0.006	3.7
<i>Massilia timonae</i>	958		43467		53705		0.002	4.4
unclassified <i>Enterobacteriaceae</i>	5826		73487		23236		0.017	4.5
unclassified <i>Haemophilus</i>	11272		6051		870		0.023	3.7

Table 1 (continued)

unclassified <i>Pseudomonadota</i>	373	1598	1448	0.042	2.8
unclassified <i>Oxalobacteraceae</i>	112	5359	5801	0.004	3.5
unclassified <i>Betaproteobacteria</i>	161	2393	4150	0.005	3.3

	*species less frequent in patients (< 65 yo or ≥ 65 yo), as compared to age-matched controls.
	*species more representative in patients (< 65 yo or ≥ 65 yo), as compared to age-matched controls.
Bold red values	*species less frequent in cases or controls older than ≥ 65 yo or < 65 yo, respectively.
Bold green values	*species most frequent in cases or controls older than ≥ 65 yo or < 65 yo, respectively.

Pulmonary microbiota composition and LEfSe results according to patient category and age. Values reported for taxa correspond to normalized and scaled relative abundances used as input for LEfSe analysis. Linear discriminant analysis (LDA) scores indicate the effect size of taxa differentially enriched among groups, as identified by non-parametric Krustal-Wallis sum rank statistical testing. Specifically, genomic deoxyribonucleic acid (DNA) was extracted (QIAmp® DNA Microbiome kit-Qiagen, Milan, Italy) and then quantified (Invitrogen Qubit 4 User Manual-Thermo Fisher, Monza, Italy). The bacterial 16S DNA libraries were prepared using the Microbiota solution A kit (Arrow Diagnostics srl, Genoa, Italy). Sequencing analysis was performed by MiSeq platform (MiSeq Reagent Nano Kit v2 (500-cycles)) and using Phix as internal standard (Illumina Inc., San Diego, CA, USA). Raw sequences were processed by MicrobAT software (SmartSeq srl, Novara, Italy, v.1.2.2 based on the Ribosomal Database Project (RDP) database (v. 11.4)). Statistical comparison for different features was performed using MicrobiomeAnalyst software

Abbreviations: SOFA Sequential Organ Failure Assessment score, IMV invasive mechanical ventilation, n-IMV non-invasive mechanical ventilation, ICU intensive care unit, BAL bronchoalveolar lavage, COT conventional oxygen therapy, HFNO high flow oxygen therapy, PaO₂/FIO₂ ratio of the partial pressure of arterial oxygen (PaO₂) to the fraction of inspired oxygen (FIO₂), pCO₂ partial pressure of arterial carbon dioxide, yo years old, LDA logarithmic discriminant analysis, LEfSe Linear Discriminant Analysis Effect Size, sp species

in Table 1, showing, as expected, worse respiratory and hemodynamic parameters in ICU-patients, as compared to non-ICU patients. LEfSe analysis was performed on normalized and scaled relative abundance data. The following pathogens were isolated from BALs: i) Gram-negative bacteria (i.e. *Serratia marcescens*, *Acinetobacter baumannii*, *E. coli*, *Pseudomonas aeruginosa*, *Stenothrophomonas maltophilia* and carbapenemase-producing *Klebsiella pneumoniae*) (n. 10 BALs from ICU-patients and n. 7 from non-ICU patients); ii) Gram-positive pathogens (i.e. *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus* sp.) (n. 3 BALs from non-ICU patients); iii) while *Aspergillus fumigatus* was isolated from one ICU-patient and one non-ICU subject.

With regards to pulmonary microbiota, a full description of phyla and species is reported in Table 1. Specifically, *Bacillota* and *Pseudomonadota* were the most prevalent phyla among cases and controls, without intergroup differences (*p*-values 0.742 and 0.474, respectively) (Table 1); while *Bacteroidota* and *Verrucomicrobiota* were more copious in ICU- and non-ICU patients above 65 years, as compared to controls and younger cases (*p*-values 0.050 and 0.030, respectively) (Table 1).

Focusing on species, some additional differences were detected. In fact, not only *Bacteroides* sp. (belonging to *Bacteroidota*) and *Akkermanansia* sp. (belonging to

Verrucomicrobiota), but also *Blautia* sp. (belonging to *Bacillota*) and *Klebsiella* sp. (belonging to *Pseudomonadota*) were more copious in older patients, regardless of gender and clinical severity (Table 1).

On the contrary, *Gemella haemolysans* (belonging to *Bacillota*), *Oxalobacteraceae* (belonging to *Proteobacteria*) and *Veillonella dispar* (belonging to *Firmicutes*) were less abundant in older ICU- and non-ICU patients, regardless of gender (*p*-values 0.034, 0.047, 0.017 and 0.012, respectively); while, *Massilia timonae* (belonging to *Pseudomonadota*) was decreased in ICU-patients, regardless of age and gender, and in older non-ICU cases solely (*p*-value 0.026) (Table 1).

Alpha- and beta-diversity of microbiota, stratified by disease severity and age, is reported in Fig. 1. As described above, a higher mean of observed species was identified in ICU- and non-ICU patients, both older than 65 years, as compared to age-matched controls and younger cases (*p*-value 0.020).

To our knowledge, little is known about the lung microbiota of pneumonia patients. Our results confirm recent findings suggesting that the lung is not sterile, as once thought, but rather hosts its own microbial flora [2]. This presence seems to be attributed to the continuous movement of fluids and air through the oral cavity, which generates biologically significant aerosols translocating into patients' airways [7, 10].

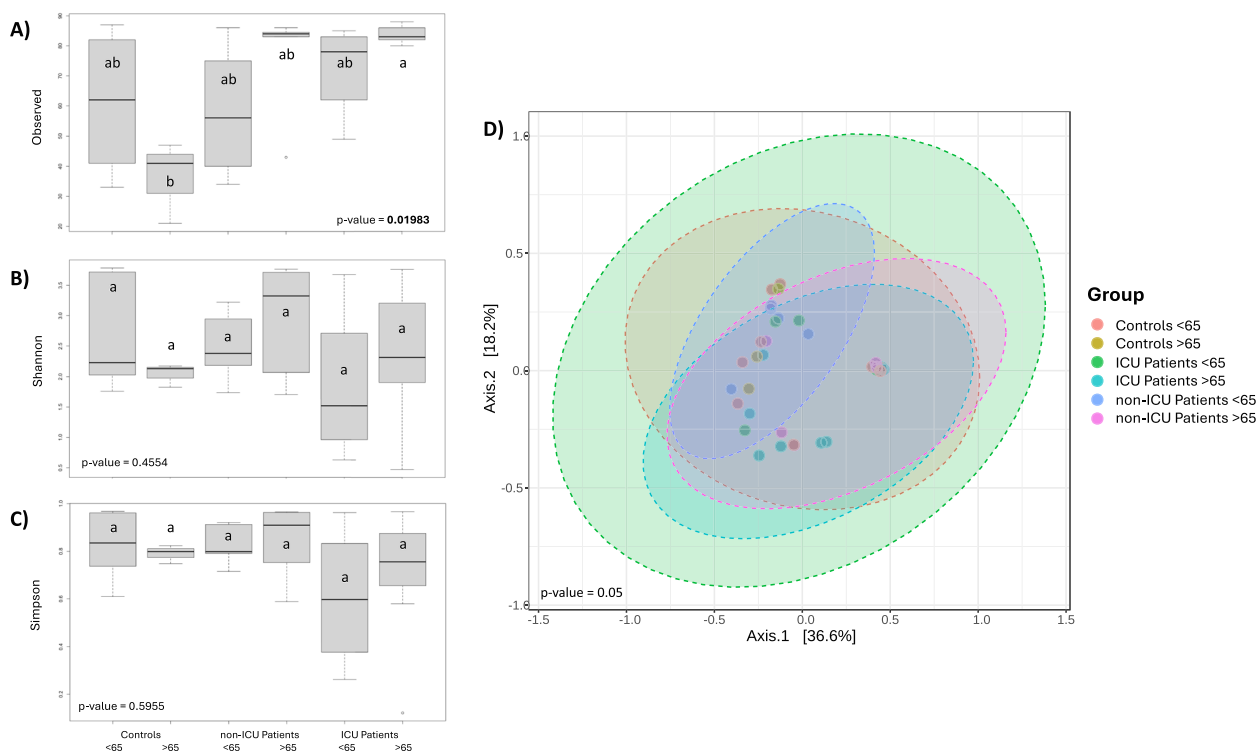


Fig. 1 Alpha diversity and beta diversity considering patient category (i.e., ICU- and non-ICU patients and according to age). Alpha diversity, defined by the total number of species (observed) and the abundances of the species (evenness) (Shannon and Simpson indices), described the diversity of the considered community (phyloseq package). Data regarding relative abundances and alpha diversity were, previously tested for normality (Shapiro’s test) and homogeneity of variances (Levene’s test), and then analyzed using one-way nonparametric tests (permutation test for the parameter “observed” and Kruskal–Wallis’ test for phylum relative abundances, Shannon and Simpson indexes). Beta-diversity described the diversity between the considered microbial communities (PERMANOVA, using the phyloseq package). As anticipated, both diversities were measured using Observed, Shannon and Simpson indexes and represented as boxplots. Each boxplot represents the different distribution of a group within Sample-Type class: **A** Observed species (p -value 0.020); **B** Shannon index (p -value 0.455); **C** Simpson index (p -value 0.596); **D** comparison by PCoA of the distance (with the Bray–Curtis distance-based method) of the different samples. *Abbreviations:* ICU: Intensive Care Unit; PCoA: Principal Coordinates Analysis

In our study, the predominant phyla were due to *Bacillota* and *Pseudomonadota*, as reported in previous studies enrolling healthy volunteers [1, 2, 10]; while *Bacteroidota* and *Verrucomicrobiota* were more abundant in older ICU- and non-ICU cases, confirming a significant dysbiosis in patients affected by pneumonia, with more pronounced differences observed in older patients.

Interestingly, *Massilia timonae*, an aerobic, non-fermenting Gram-negative bacterium, first described in 1998 in healthy lungs [10], was the only species that showed a lower relative abundance at the group level, regardless of clinical severity. In fact, a low expression of *M. timonae* has been previously associated with a prior exposure to antibiotics, targeting Gram-negative bacteria, often administrated during pneumonia [10]. So doing, the low levels of *M. timonae*, as newly described by our study, may increase patients’ susceptibility to pulmonary infections and shed light on how host condition and critical care interventions may influence the lung microbiota.

Despite these promising results, some weaknesses need to be declared. First, the relatively small sample size and single-center profile may limit the ability to detect potential intra- and inter-group differences. Second, the ‘control group’ was composed of cadavers, relatively younger than cases and not always comparable to age-matched healthy volunteers. However, all samples were collected within 1 h after death with the aim to minimize potential significant changes to the lower airway microbiota following arrest. Additionally, healthy volunteers were not selected as controls because performing BAL in this cohort would not be ethically justifiable. Third, it’s widely acknowledged that factors such as antibiotics, nutrition, proton pump inhibitor use, and selective decontamination can impact microbiota. However, due to the limited number of patients included in the study, it was not possible to investigate any potential correlation with microbiota. Fifth, as shown in Table 1, males were predominant as

compared to females, reflecting the typical ICU population. However, this unbalanced distribution could be a weakness in data interpretation.

Fourth, according to our methodology, only some species and phyla were analyzed. Therefore, we cannot exclude the presence of other relevant species or phyla in lung microbiota.

In conclusion, our study showed that pneumonia can negatively affect lung microbiota, especially in older patients (as compared to younger cases), while the impairment is less remarkable according to clinical severity and gender. Indeed, microbiota phenotyping may offer novel insights into the pathophysiology of pneumonia and pulmonary dysbiosis.

This study has inherent limitations related to its exploratory design. The small sample size and single-center setting limit statistical power and warrant cautious interpretation of the findings, which should be considered hypothesis-generating. High inter-individual variability was observed, a recognized feature of microbiome data, particularly in low-biomass environments such as the lung. Although non-parametric statistical approaches and LEfSe analysis were applied to mitigate the impact of non-normal distributions, variability may still have influenced the results.

The control group consisted of younger cadaveric subjects; while bronchoalveolar samples were collected within one hour after death to minimize post-mortem microbial shifts, age-related and early post-mortem effects cannot be fully excluded. Moreover, detailed stratification by antibiotic exposure prior to BAL collection was not feasible, representing a potential confounding factor, especially in ICU patients. Larger, multicenter studies with comprehensive clinical metadata are needed to confirm and extend these observations.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s44158-026-00363-1>.

Supplementary Material 1.

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Authors' contributions

ABr, EG, AR, CP, FL, EB, IA, PN and AB: conception and design of the work, acquisition and interpretation of data, and drafting of the work. ABr, EG, AR, CP, FL, EB, IA, PN and AB: design of the work, acquisition of data. ABr, EG, AR, CP, FL, EB, IA, PN and AB: acquisition of data. ABr, EG, AR, CP, FL, EB, IA, PN and AB: analysis and interpretation of data. ABr, EG, AR, CP, FL, EB, IA, PN and AB: revision of the draft. ABr, EG, EB, AB and PN: design of the work and substantial revision of the draft.

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Data availability

Individual participant data that underlie the results reported in this article, after de-identification, data dictionary, study protocol, statistical analysis plan, informed consent form, and analytic code will be available to any researchers who provide a methodologically sound proposal, immediately following publication and without end date. Proposals should be directed to the corresponding author. To gain access, data requestors will need to sign a data access agreement.

Declarations

Ethics approval and consent to participate

The study was designed following the Declaration of Helsinki and the protocol was first approved by the Ethics Committee of the Azienda Ospedaliera Universitaria Dulbecco of Catanzaro (protocol approval n. 113, Aprile 20, 2023). Patient consent was obtained according to the national regulations.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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