國立臺灣大學醫學院臨床醫學研究所

博士論文

Graduate Institute of Clinical Medicine
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Doctoral dissertation

慢性阻塞性肺病及支氣管擴張症的肺微生物菌叢基因體研究

Lung microbiome research in chronic obstructive pulmonary disease and bronchiectasis

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口試委員會審訂書

慢性阻塞性肺病及支氣管擴張症

的肺微生物菌叢基因體研究

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本論文係陳彥甫君（學號 D04421012）在國立臺灣大學臨床醫學研究所完成之博士學位論文，於民國 113 年 11 月 29 日承下列考試委員審查通過及口試及格，特此證明

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王弘毅

系主任、所長 林千浩（簽名）

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致謝

首先，我衷心感謝我的指導教授—余忠仁院長，余教授帶領我進入 lung microbiome 微菌研究這個充滿挑戰與無限可能的領域。在博士班的學習過程中，余教授每月召開研究會議，不斷啟發與引導我去找尋答案。我的研究旅程始於他給我的一個詞語—“Microbiota”，並開始學會如何建立研究的 Cohorts、尋求合作及建立團隊（teamwork）、爭取國科會計畫(Funding)(至今已連續 7 年通過多年期計畫)，以及與國際學者展開合作(Collaboration)。在研究設計與實驗過程中，余教授給予我無數寶貴的建議，這些經驗指導成為我學術成長的重要基石。對於老師的悉心栽培與鼓勵，我心懷無限感激。

此外，我必須特別致意於王鶴健教授和簡榮彥教授。他們作為我研究會議中的重要成員，對我的研究設計和論文撰寫提供了許多指導和寶貴建議。同時，他們協助台灣胸腔暨重症學會建立了本土第一個大型支氣管擴張症研究團隊：台灣支氣管擴張症研究聯盟（Taiwan Bronchiectasis Research Collaboration, TBARC）cohort，為我的研究提供了進一步的臨床結果驗證。另外，我也誠摯感謝博士班口試委員：台灣微菌聯盟(Taiwan Microbiota Consortium)的理事長吳俊穎教授、臺北醫學大學李岡遠副校長和臨床醫學研究所王弘毅教授，感謝他們在我的博士資格考試和論文諮詢委員會階段提供研究上的指導，這些寶貴的意見，使我在呼吸道微生物菌叢領域的研究更加完善。

我要特別感謝台大醫院雲林分院教學研究部的在經費及研究設備上的支持，為後續國科會計畫的成功奠定了堅實的基礎。同時，我深深感激王敬媛和劉育珊兩位助理的辛勤付出，她們在研究收案和計畫申請中做出了極大的貢獻；還有精準醫學中心的陳盈吟，她為初步的臨床檢體處理上付出了許多心力，甚至常常因研究而加班。此外，我要向總院實驗室的合作夥伴—口腔生物科學研究所侯欣翰老師、台大總院內科助理呂鍇任和洪政慈—表達由衷的謝意，長期以來他們協助進行所有的檢體分析與儲存，是我呼吸道微菌研究最重要的核心支持。另外，還要感謝台大癌醫中心影像醫學科的簡寧醫師及台大醫工所的羅國榮博士在肺部影像的分析上大力協助。最後，特別感謝國衛院的廖玉潔老師和林介華老師，他們在 bioinformatics 方面的鼎力相助，讓 lung microbiome 分析得以順利推進完成，並促成了文章的成功發表。

此外，感謝臨床醫學研究所的師長及同學這幾年來的指導與分享，不僅拓寬了我的學術視野，也讓我感受到不同領域間的扶持與激勵。特別感謝樹金忠學長及導師高嘉宏教授，他們分享了許多寶貴的研究建議，這些經驗成為我繼續前行的動力。你們的支援與協助，讓我能夠在完善的學術環境中專注於研究，再次表達由衷的感謝。

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陳彥甫 謹識

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中文摘要

背景：慢性阻塞性肺病（COPD）和支氣管擴張症（Bronchiectasis, BE）都是異質性肺病，具有複雜的臨床和病理特徵。這些特徵的共存會加劇臨床症狀、強化炎症，並導致疾病惡化及較差的預後。當支氣管擴張症患者合併固定氣流阻塞（fixed airflow obstruction, FAO）時，通常同時滿足阻塞性肺功能測試標準及支氣管擴張症的影像結構性診斷。這表明這群患者符合近期所提出的「慢性阻塞性肺病-支氣管擴張症關聯症」（COPD-BE association）這一新的診斷群體。然而，肺微生物菌叢基因體(lung microbiome)在 COPD-BE 關聯症中的作用仍然不明確。本研究旨在利用支氣管肺泡灌洗液(Bronchoalveolar lavage, BAL)樣本，探討肺微生物菌叢基因體在慢性阻塞性肺病、支氣管擴張症及同時符合這兩種診斷的患者中的角色。此外，我們通過基於 ROSE（放射學 Radiology、阻塞 Obstruction、肺功能測試 Spirometry 和暴露 Exposure）標準對支氣管擴張症患者進行分類，以評估氣道炎症標誌物的臨床意義及其相關的臨床預後。

方法：我們在台灣進行了一項前瞻性觀察研究，招募患有支氣管擴張症或慢性阻塞性肺病的患者。我們分析了這些患者的肺微生物菌叢基因體，並評估其氣道發炎的生物標誌物。同時，我們收集了支氣管肺泡灌洗樣本，進行 16S 核糖體核糖核酸基因定序分析。此外，我們還通過收集兩個支氣管擴張症世代研究來驗證 ROSE 標準在支氣管擴張症患者中的應用，包括一個前瞻性單中心世代和一個回顧性多中心世代，以評估 COPD-BE 關聯症的臨床意義和預後，並與其他有或沒有固定氣流阻塞（FAO）的支氣管擴張患者進行比較。

結果：在觀察性世代研究中，我們收入了 181 名患者，其中包括 86 名慢性阻塞性肺病患者、46 名支氣管擴張症患者和 49 名經肺功能檢查確認的支氣管擴張症合併固定氣流阻塞(BE-FAO)患者。我們發現無論有無合併氣流阻塞，支氣管擴張症患者的微生物群特徵相似，表現為 α 多樣性降低和變形菌門（*Proteobacteria*）占主導地位，與展現更多厚壁菌門（*Firmicutes*）、更大多樣性和更多共生類群的慢性阻塞性肺病患者顯著不同。此外，與無固定氣流阻塞的 COPD 和 BE 相比，患有固定氣流阻塞的支氣管擴張症(BE-FAO)患者顯示出更嚴重的疾病和更高的惡化風險。我們發現綠膿桿菌（*Pseudomonas aeruginosa*）的存在與氣道嗜中性球發炎症的增加顯著相關，包括白介素（IL）-1β、IL-8和腫瘤壞死因子α（TNF-α），以及更高的支氣管擴張症嚴重程度，

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這可能導致惡化風險的增加。此外，在患有固定氣流阻塞的支氣管擴張症(BE-FAO)患者中，根據吸菸史的有無，使用 ROSE 標準將個體分類為 ROSE（+）或 ROSE（-）。這一分類突顯了 ROSE（-）和 ROSE（+）患者之間在臨床特徵、炎症特徵和微生物群細微變化上的差異，暗示在合併有氣流阻塞的支氣管擴張症(BE-FAO)患者群體中存在多樣的內表型(Endotypes)。

我們又在台灣人群中進行了一項世代分析，將 147 名參與者的前瞻性世代與 574 名參與者的多中心回顧性世代結合。使用 ROSE 標準，我們發現約有 16.5%的參與者具有 COPD-BE 關聯症(前瞻性世代族群中為 22.4%，回顧性世代族群中為 14.9%），主要集中在年長的男性患者中。這些患者與無氣流阻塞的支氣管擴張症患者相比，他們的呼吸困難指數上升，臨床上合併有慢性阻塞性肺病診斷率較高，使用吸入治療的比例也增加。值得注意的是，儘管具有 COPD-BE 關聯患者和無吸菸的支氣管擴張症合併固定氣流阻塞(nonsmoking BE-FAO)患者在臨床症狀、肺功能和疾病嚴重性上表現相似，但在氣道的微生物學上略有不同。此外，具有 COPD-BE 關聯的患者在調整干擾因素後，仍顯示出其有顯著較高的急性惡化和住院的風險，強調 COPD-BE 關聯症的患者臨床預後較其他群體更差。

結論：根據 ROSE 標準進行定義，患有支氣管擴張症合併固定氣流阻塞（BE-FAO）的患者可能表現出兩種不同的內表型。這些內表型的特徵為疾病嚴重程度更高，其肺微生物菌叢基因體更接近於沒有固定氣流阻塞的支氣管擴張症患者，而不是慢性阻塞性肺患者。綠膿桿菌定殖(colonization)與氣道嗜中性球發炎症增加之間的顯著相關性以及疾病嚴重程度，突顯了氣道微生物菌叢的臨床意義。此外，我們發現使用 ROSE 標準可以有效地找出東亞人群中的具有 COPD-BE 關聯症患者，顯示出相較於其他支氣管擴張症群體，其未來急性惡化的風險顯著的增加。這一發現強調了這些肺微生物菌叢基因體在支氣管擴張症合併固定氣流阻塞的疾病進展與其在急性惡化中的潛在作用。未來的研究亟需深入了解支氣管擴張症的進展，特別是在合併固定氣流阻塞的族群。

關鍵詞：16S 核糖体核糖核酸基因定序、支氣管擴張症、慢性阻塞性肺病-支氣管擴張症關聯症、固定氣流阻塞、肺微生物菌叢基因體、ROSE 標準

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ABSTRACT

Background: Chronic obstructive pulmonary disease (COPD) and bronchiectasis (BE) are both heterogeneous lung diseases characterized by complex clinical and pathological features. The coexistence of these features exacerbates symptoms, intensifies inflammation, and worsens prognosis compared to either condition alone. Patients with bronchiectasis and fixed airflow obstruction (FAO) meet both the obstructive spirometry criteria for COPD and the structural diagnosis of bronchiectasis, reflecting the recently proposed COPD-BE association. However, the role of the lung microbiome in the COPD-BE association remains unclear. This study aimed to investigate the role of the lung microbiome in patients with COPD, bronchiectasis, and those who meet both diagnoses, using bronchoalveolar lavage (BAL) samples. Additionally, we sought to evaluate airway inflammatory markers, their clinical significance, and outcomes by categorizing bronchiectasis patients based on the ROSE (Radiology, Obstruction, Spirometry, and Exposure) criteria.

Methods: We conducted a prospective observational study in Taiwan, enrolling patients with either bronchiectasis or COPD. To analyze the lung microbiome and assess inflammatory markers, BAL samples were collected for 16S rRNA gene sequencing. Additionally, we validated the ROSE criteria in two bronchiectasis cohorts—a prospective single-center cohort and a retrospective multicenter cohort in Taiwan—to assess the clinical implications and clinical outcomes of the COPD-BE association compared to other groups with or without FAO.

Results: The study cohort comprised 181 patients: 86 with COPD, 46 with bronchiectasis, and 49 with bronchiectasis and FAO, confirmed by spirometry. Patients with bronchiectasis, regardless of FAO, had similar microbiome profiles characterized by reduced alpha diversity and a predominance of *Proteobacteria*, which were distinctly different from COPD patients who exhibited more *Firmicutes*, greater diversity, and more commensal taxa. Furthermore, compared to COPD and BE without FAO, BE with FAO showed more severe disease and a higher risk of exacerbations. A significant correlation was found between the presence of

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Pseudomonas aeruginosa and increased airway neutrophilic inflammation, including Interleukin (IL)-1β, IL-8, and tumor necrosis factor-alpha (TNF)-α, as well as higher bronchiectasis severity, which may contribute to an increased risk of exacerbations. In BE patients with FAO, the ROSE criteria were employed to classify individuals as either ROSE (+) or ROSE (-) based on smoking history. This classification highlighted differences in clinical features, inflammatory profiles, and slight microbiome variations between ROSE (-) and ROSE (+) patients, suggesting diverse endotypes within the BE with FAO group.

An integrated cohort analysis was conducted within a Taiwanese demographic, combining a prospective cohort of 147 participants with a multicenter retrospective cohort of 574 participants. Using the ROSE criteria, we found that 16.5% of participants had a COPD-BE association (22.4% in the prospective cohort and 14.9% in the retrospective cohort), predominantly among older male patients. These patients had escalated dyspnea scores, higher COPD diagnosis rates, and increased use of inhalation therapies compared to those without FAO. Notably, patients with a COPD-BE association and nonsmoking BE with FAO displayed similar clinical symptoms, pulmonary function, and disease severity but differed slightly in airway microbiology. Furthermore, patients with a COPD-BE association had significantly higher risks of exacerbations and hospitalizations, even after adjusting for confounding factors, highlighting poorer clinical outcomes compared to other groups.

Conclusion: Patients with bronchiectasis and FAO may exhibit two distinct endotypes, as defined by the ROSE criteria. These endotypes are characterized by greater disease severity and a lung microbiome that is more similar to that of bronchiectasis patients without FAO than to those with COPD. The significant correlation between *Pseudomonas aeruginosa* colonization and increased airway neutrophilic inflammation, along with disease severity, underscores the clinical relevance of microbial patterns. Additionally, we found that the ROSE criteria effectively identify the COPD-BE association in East Asian populations, highlighting a significantly higher risk of future exacerbations compared to other bronchiectasis groups.

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This finding reinforces the potential role of this lung microbiome in the progression and exacerbations of bronchiectasis with FAO. Future research is warranted to better understand the progression of bronchiectasis, particularly in subgroups with FAO.

Keywords: 16S rRNA Gene Sequencing, Bronchiectasis, COPD-BE Association, Fixed Airflow Obstruction, Lung Microbiome, ROSE Criteria

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LIST OF ABBREVIATIONS

1.	ABPA	Allergic bronchopulmonary aspergillosis
2.	aOR	Adjusted Odds Ratio
3.	ASV	Amplicon sequence variants
4.	ATS	American Thoracic Society
5.	BALF	Bronchoalveolar lavage fluid
6.	BCW	Bronchoscope channel washing
7.	BE	Bronchiectasis
8.	BE-FAO	Bronchiectasis with fixed airflow obstruction
9.	BPD	Bronchopulmonary dysplasia
10.	BSI	Bronchiectasis Severity Index
11.	BMI	Body mass index
12.	CAT	Chronic obstructive pulmonary disease Assessment Test
13.	CE	Capillary electrophoresis
14.	CF	Cystic fibrosis
15.	COPD	Chronic obstructive pulmonary disease
16.	CRP	C-reactive protein
17.	CXR	Chest X-rays

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18.	DADA2	Divisive Amplicon Denoising Algorithm 2
19.	dNTPs	Deoxyribonucleotide triphosphates (dNTPs)
20.	DNA	Deoxyribonucleic acid
21.	EKC	Extraction kit control
22.	EMBARC	European Bronchiectasis Registry
23.	ERS	European Respiratory Society
24.	FAO	Fixed airflow obstruction;
25.	FDR	False discovery rate
26.	FeNO	Fraction of Exhaled Nitric Oxide
27.	FEV_1	Forced expiratory volume in 1s
28.	FEV_1/FVC	Forced expiratory volume in 1s/Forced vital capacity
29.	FVC	Forced vital capacity
30.	GERD	Gastroesophageal reflux disease
31.	GOLD	Global Initiative for Chronic Obstructive Pulmonary Disease
32.	HMP	Human Microbiome Project
33.	HRCT	High-resolution computed tomography
34.	HU	Hounsfield unit
35.	ICS	Inhaled corticosteroid

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36.	IL-1 β	Interleukin [IL]-1 β
37.	IL-6	Interleukin [IL]-6
38.	IL-8	Interleukin [IL]-8
39.	ILD	Interstitial Lung Disease
40.	IPF	Idiopathic pulmonary fibrosis.
41.	IQR	Interquartile range
42.	IRR	Incidence rate ratio
43.	ITS	Internal Transcribed Spacer
44.	LABA	Long-acting β2 Sympathomimetic Agonists
45.	LAMA	Long-acting muscarinic antagonist
46.	LAV	Low-attenuation volume
47.	LLL	Left lower lobe
48.	LRT	Lower respiratory tract
49.	LUL	Left upper lobe
50.	MCP-1	Monocyte chemoattractant protein-1
51.	mMRC	Modified Medical Research Council
52.	MPO	Myeloperoxidase
53.	NA	Not Available

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54. NARLabs National Applied Research Laboratories

55. NCBI National Center for Biotechnology Information

56. NCHC High-performance Computing

57. NETs Neutrophil extracellular traps

58. NGS Next generation sequencing

59. NIH National Institutes of Health

60. NLR Neutrophil-lymphocyte ratio

61. NSC Normal saline control

62. NTC Non-template control

63. NTM Non-tuberculosis mycobacteria

64. NTUH National Taiwan University Hospital

65. OR Odds Ratio

66. OTU Operational taxonomic units

67. OWC Oral washing control

68. PBS Phosphate-buffered saline

69. PCoA Principal co-ordinates analysis

70. PCR Polymerase chain reaction

71. PERMANOVA Permutational multivariate analysis of variance

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72.	QC	Quality control
73.	QIIME	Quantitative Insights Into Microbial Ecology
74.	RLL	Right lower lobe.
75.	RML	Right middle lobe.
76.	RNA	Ribonucleic acid
77.	ROSE	Radiology, Obstruction, Symptoms, Exposure
78.	rRNA	Ribosomal Ribonucleic acid
79.	RUL	Right upper lobe
80.	SBS	Sequencing by synthesis
81.	SD	Standard deviation
82.	SRA	Sequence Read Archive
83.	TB	Tuberculosis
84.	TBARC	Taiwan Bronchiectasis Research Collaboration
85.	TNF-α	Tumor necrosis factor [TNF]- α
86.	TOLD	Taiwan Obstructive Lung Disease
87.	URT	Upper respiratory tract
88.	Vit D3	Vitamin D3
89.	WGS	Whole genome sequencing

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Chapter 1. Background

1.1 History of lung microbiome

With the advent of the Human Microbiome Project (HMP), our understanding of the human body's microbiome has significantly expanded. Launched by the National Institutes of Health (NIH) in 2007 [1], the HMP aimed to identify and characterize the microorganisms associated with both healthy and diseased human bodies. It provided a comprehensive map of microbial communities inhabiting various regions such as the skin, gut and mouth [1,2]. The human microbiome, in general, includes all microorganisms and their genetic material (including homologous sequences) in a specific habitat at a specific time [3,4]. The lung microbiome, once thought to be sterile, has now been recognized as a dynamic ecosystem of bacteria, archaea, fungi, and viruses that reside in the respiratory tract [5] **(Figure 1).**

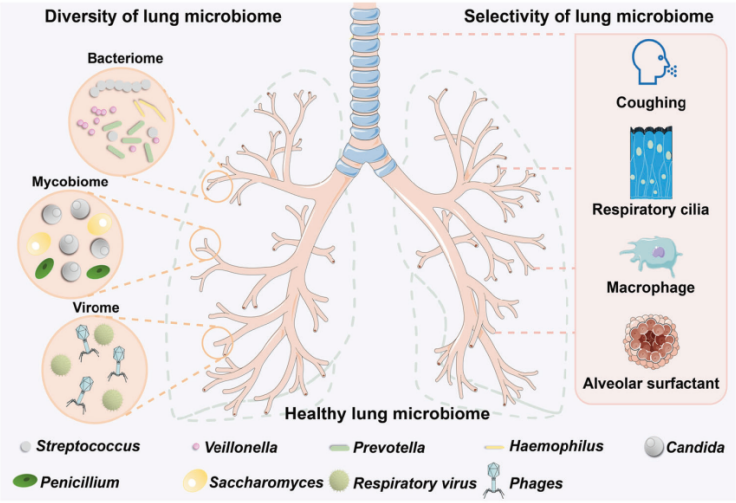

Figure 1. Composition of the lung microbiome. In contrast to the highly diverse microbial communities found in the gut and oropharyngeal regions, the lung microbiome hosts fewer resident microorganisms. However, this does not imply that it is uniform. The lung microbiome consists of three main components: the bacteriome, mycobiome, and virome. Adapted from [Reference 3].

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1.2 NGS and detectable lung microbiome

In clinical microbiology, culture-dependent methods remain the gold standard for detecting pathogens. However, isolating specific bacteria from complex mixtures requires time and expertise, as different microorganisms need unique nutrients, atmospheric conditions (aerobic, anaerobic, micro-aerophilic), and temperatures for growth. Identification through these methods can be slow, often taking 24–72 hours for primary growth, with some bacteria requiring weeks for sufficient growth [6,7].

Recently, molecular diagnostics based on nucleic acids, proteins, and metabolites have emerged to accelerate microbial identification. The advancements in next-generation sequencing (NGS) technology have revolutionized our understanding of the lung microbiome, challenging the old belief that healthy lungs are sterile [7,8,9]. Both bacterial Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA) can now be used to detect and identify microbes in various samples, regardless of growth conditions. These nucleic acids act as templates for amplification through methods like Polymerase chain reaction (PCR), hybridization (e.g., microarrays), and sequencing. PCR, the first culture-independent diagnostic tool, enables the detection of even trace amounts of microorganisms that would otherwise be undetectable. The use of PCR with species-specific primers offers high sensitivity for identifying pathogens, making it the gold

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standard for quickly and cost-effectively detecting viruses, parasites, and bacteria [10,11,12]. Techniques such as 16S ribosomal RNA (rRNA) gene sequencing, fungal amplicons and shotgun metagenomics allow for high-resolution analysis of microbial DNA, revealing diverse microbial communities in the respiratory tract, especially in various lung diseases **(Figure 2)** [13]

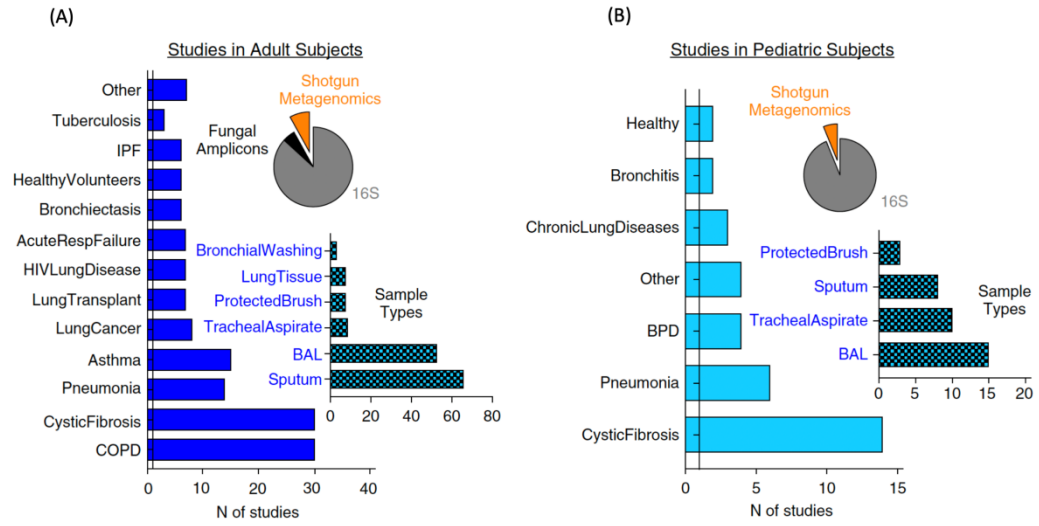

Figure 2. **Overview of published lung microbiome research (2015–2018)** (A) The bar graph presents an analysis of studies conducted in adults, categorized by disease. It shows that chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) are the most frequently studied conditions. Insets: Amplicon sequencing, targeting 16S rRNA or fungal genomes, was the predominant sequencing method used in 91% of studies; sputum and bronchoalveolar lavage fluid (BALF) were the most frequently analyzed sample types. (B) Similarly, the bar graph highlights studies involving pediatric subjects, also categorized by disease, where CF and pneumonia emerged as the most common areas of focus. Insets: 16S rRNA gene sequencing was employed in 93% of studies, and BALF was the primary sample type. Abbreviations: BPD = bronchopulmonary dysplasia; IPF = idiopathic pulmonary fibrosis. Adapted from Reference 13.

1.2.1 16S rRNA gene sequencing

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All bacteria contain ribosomes, composed of a 50S and 30S subunit. The smaller 30S subunit includes 21 proteins and 16S ribosomal RNA. Due to its critical role, the 16S rRNA gene is highly conserved across bacteria, allowing PCR primers to amplify this gene in virtually all bacterial species. Additionally, the gene's nine hypervariable regions (V1–V9) [13,14] provide a molecular fingerprint for identifying different organisms, making it the gold standard for identifying taxonomic units via high-throughput sequencing. Targeting specific region sequences (usually V3–V4) increases the resolving power for identifying bacterial taxa **(Figure 3)** [15].

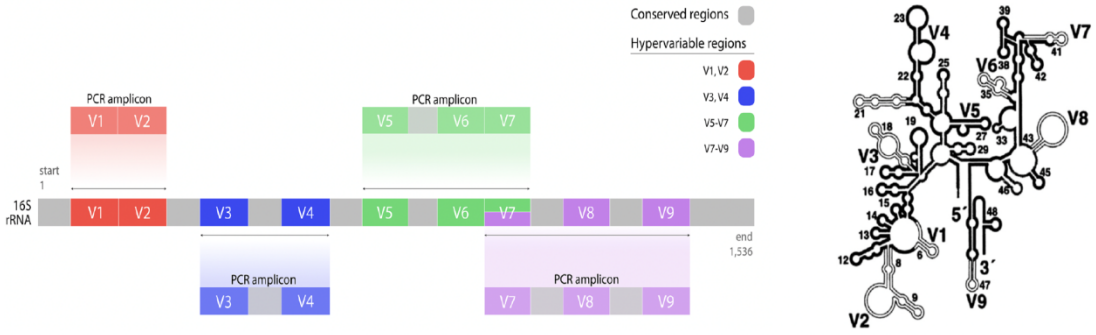

Figure 3. 16S rRNA hypervariable regions.
This figure shows the nine hypervariable regions (V1–V9) on the secondary structure of 16S rDNA. The V1–V3 and V3–V4 regions are commonly used for bacterial microbiome characterization. (Adapted from references 14 and 15)

Sequencing the PCR products from a mixed DNA sample reveals the microorganisms present, and the number of reads for each sequence indicates their relative abundance. Although this technique has been in use for decades, its application in respiratory research

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has only become common over the last 5–10 years. This method provides a comprehensive profile of all bacteria in the sample, their relative abundance, and allows for comparisons over time or between different sample groups. In case–control studies, it can help identify how clinical conditions, treatments, or infections impact the bacterial community [13].

However, there are some limitations. This technique generally offers genus-level resolution, making it difficult to pinpoint specific species. It also doesn't provide functional insights, such as metabolic pathways, which are achievable with shotgun metagenomics. The results can be influenced by differences in methodologies, such as DNA extraction methods, PCR protocols, and primer selection, which can affect subsequent analysis [16,17,18].

1.2.2 Shotgun metagenomics gene sequencing

In metagenomics, instead of amplifying a single gene, the entire mixed DNA from a sample is sequenced, eliminating the PCR steps required in the 16S rRNA approach and reducing associated biases. This approach also provides access to all functional genes of each organism within the sample, allowing the potential study of antimicrobial resistance, virulence factors, and the metabolic pathways of the micro-organisms present [13].

Metagenomics improves the resolution of bacterial identification within a sample, enabling strain-level typing and the tracking of infectious agents during outbreaks [19–

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22]. Since bacterial genomes are much larger than individual bacterial genes, this method requires significantly deeper sequencing to fully characterize a microbial community. Unlike 16S rRNA gene sequencing, which targets a single bacterial gene, metagenomics also sequences human DNA. This is especially relevant in low microbial biomass respiratory samples, where most of the sequenced reads originate from human genomic material [22–24]. Consequently, the increased costs and analytical complexity required to resolve a complex mixture of DNA sequences from a metagenome have limited the number of respiratory studies **(Figure 2)** [22, 24–27].

1.2.3 ITS1 and ITS2 gene sequencing for fungal mycobiome

Internal Transcribed Spacer (ITS) sequences are critical components of ribosomal RNA genes in eukaryotes. These non-coding regions, particularly ITS1 and ITS2, are widely used in fungal mycobiome studies due to their significant variability, which makes them highly suitable for distinguishing fungal species. The ITS1 region is located between the 18S and 5.8S rRNA genes, while ITS2 is situated between the 5.8S and 28S rRNA genes. Their variability allows for the differentiation of closely related species, making them indispensable markers for fungal taxonomy and phylogenetic studies [28, 29].

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To accurately amplify these regions, specific primers are used for PCR. Common primer sets include ITS1-F and ITS2 for the ITS1 region, and ITS3 and ITS4 for the ITS2 region. In mycobiome analysis, ITS1 and ITS2 sequencing is typically performed using high-throughput sequencing platforms like Illumina. These regions provide high-resolution data for profiling fungal communities, investigating their diversity, and understanding their ecological roles. Although ITS1 is often preferred due to its shorter amplification fragment and broader primer applicability, both ITS1 and ITS2 contribute valuable insights, depending on the specific research objectives [28,30]

1.2.4 Lung microbiome: from samples to results

To study the lung microbiome, researchers follow several key steps **(Figure 4)**. First, samples are collected (A) from the subject using culture-independent methods. Commonly, this involves induced sputum or Bronchoalveolar lavage (BAL) sampling. After collection, DNA is extracted (B) from the sample. A variety of extraction methods are available, though no single standard method is universally applied. The extracted DNA will contain genetic material from both the host and the microbiome.

Next, the extracted DNA is sequenced (C) using NGS technology. Before sequencing, specific genes, such as the bacterial 16S rRNA gene or the fungal ITS region, may be amplified using PCR. Additionally, metagenomics and metatranscriptomics

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approaches, which analyze the complete genomic or transcriptional output of the microbiome, are increasingly being employed. NGS generates large volumes of sequence data, which are processed (D) to identify the microorganisms present in the sample, typically categorized as operational taxonomic units (OTUs). Finally, this information is analyzed (E) using specialized bioinformatics algorithms, resulting in various outputs (F), ranging from organism abundance tables to visual plots that cluster samples based on microbial similarity [31]

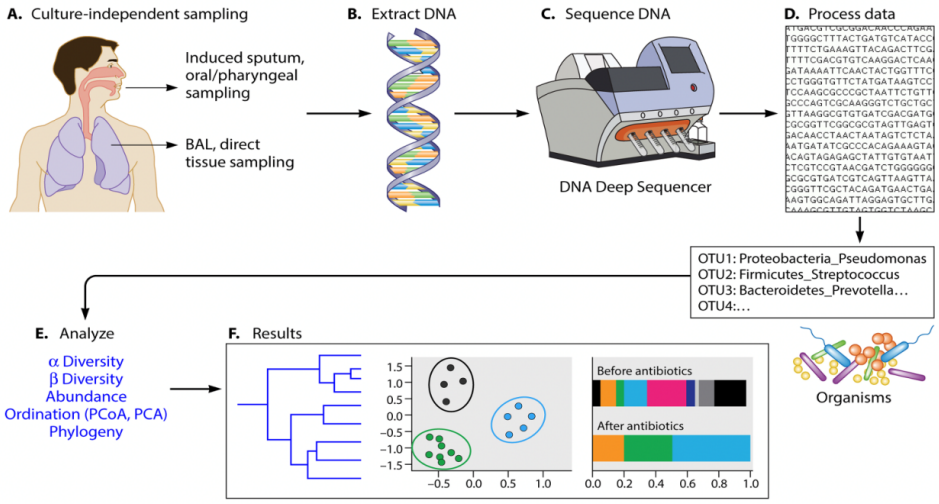

Figure 4. Lung microbiome: from sample to results.

(A) First, samples are collected from the subject using culture-independent methods. (B) DNA is then extracted from these samples, containing genetic material from both the host and the microbiome. (C) The extracted DNA is sequenced using next-generation sequencing (NGS) technology, where specific regions such as the bacterial 16S rRNA gene or the fungal ITS region may be amplified using PCR. Additionally, metagenomics and metatranscriptomics approaches, which analyze the entire genomic or transcriptional output of the microbiome, are increasingly being used. (D) The microorganisms in the sample are identified and categorized into operational taxonomic units (OTUs). (E) This data is then processed using specialized bioinformatics algorithms, (F) yielding a variety of outputs, from tables detailing organism abundance to visual plots clustering samples based on microbial similarity (Adapted from reference 31.)

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1.3 Sampling and collection of lung microbiota

The human respiratory tract is a vast and diverse ecosystem, spanning hundreds of kilometers of airways that divide fractally at least 23 times in adult lungs, resulting in an alveolar surface area of 70 m^2, which is 30 times larger than the skin and double that of the gastrointestinal tract [32,33]. It ranges from the microbe-rich pharynx to the relatively sterile alveoli, maintaining a dynamic balance of microbial immigration and elimination. The ecological and microbiological consequences of these anatomical and physiological differences are profound, influencing the burden, stability, and identity of lung bacteria. Lung bacteria, as measured by the quantification of bacterial DNA, are roughly 100-fold lower in concentration than oral bacteria [34]. The lungs are also anatomically, physiologically, immunologically, and, most importantly, ecologically distinct from the gut [34]. Sampling lung microbiota, therefore, requires careful consideration of anatomical, physiological, microbiological, and procedural factors [35].

1.3.1 Sputum sample collection

Sputum is an extracellular gel composed of water, heavily glycosylated mucins, blood, DNA, actin, inhaled toxins, particulate matter, host cells, and microbial cells along with their associated products [36]. It is produced by the mucociliary escalator, which lines the

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airway mucosa. Sputum is a critical sample type in various chronic airway diseases, including chronic obstructive pulmonary disease (COPD), asthma, cystic fibrosis (CF) and bronchiectasis [35,37,38]. Sputum culture is a standard procedure in the management of many respiratory diseases, often used to guide targeted therapy against infecting microorganisms [34,35]. However, it is not always clear which part of the respiratory tract the sputum sample represents, and because sputum is expectorated, there is a risk of salivary contamination. In patients with more severe disease, bronchoscopy may be contraindicated, making sputum the only viable method for sampling the lower respiratory tract. One of the key advantages of sputum sampling over bronchoscopic methods is that it can be done more frequently, does not require sedation or anesthesia, and is non-invasive [35].

Some patients, such as those with COPD or even bronchiectasis, may not spontaneously produce sputum. Additionally, patients who are sputum producers may stop producing sputum after starting treatment, potentially causing the loss of a key sampling method during a longitudinal study involving an intervention [38]. In cases where spontaneous sputum production is absent, induced sputum serves as an alternative. This procedure involves having the patient inhale a hypertonic saline solution, which stimulates the respiratory tract and encourages the patient to huff and cough, helping to

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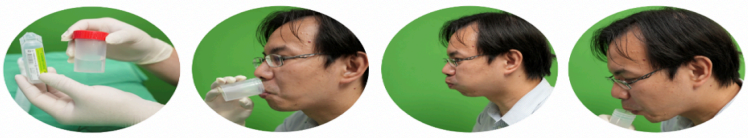
loosen and expel respiratory secretions [35].

The following is our sputum induction protocol for study participants (Figure 5).

Figure 5. Sputum induction protocol

1.Ask the patient to wear gloves and gargle with 10 ml of 0.9% saline solution for 1 minute, then spit into a 50 ml sterile container. Place the mouth gargle sample on ice and send it to the laboratory.

2.To prevent contamination before induction, ask the patient to gargle with 0.12% chlorhexidine mouthwash for 1 minute and discard the solution.



3.To avoid the residual chlorhexidine mouthwash affecting subsequent respiratory sample collection, ask the patient to gargle again with 10 ml of 0.9% saline solution for 1 minute, then discard.

4.Have the patient wear a nasal clip and inhale nebulized saline (0.9% or 3%) using a mask. (Nebulized saline can be delivered via a nebulizer with a minimum output of 2.5 L/min or a mesh portable nebulizer.)

5.Ask the patient to use the "Huff" cough technique, coughing up sputum every 5 minutes, collecting 1-3 samples in total (over 5-15 minutes) with 3-5 ml sputum sample

(1) Before each sputum collection, have the patient spit out saliva into a 50 ml sterile collection tube.

(2) Using the "Huff" cough technique, the patient should cough up the induced sputum into a 50 ml sterile collection tube.

(Note: Pause the timer while the patient collects sputum using the "Huff" technique.)

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1.3.2 Bronchoalveolar lavage (BAL) sample collection

In addition to sputum, bronchoscopically acquired respiratory specimens are among the most commonly used sample types in lung microbiome research. Bronchoscopic sampling has been utilized clinically for decades to diagnose infections, and while it is not a perfect standard for eliminating pharyngeal contamination in lower respiratory tract (LRT) microbiota sampling, it is widely accepted. Although more invasive and costly compared to sputum collection, bronchoscopic sampling offers several practical advantages. The procedure is safe, well-established, and familiar to clinicians, allowing it to be performed repeatedly in the same subject. Unlike surgical sampling, bronchoscopy typically requires only conscious sedation in adults rather than general anesthesia, and it avoids microbiological confounding factors like perioperative antibiotics [9]. This makes bronchoscopic sampling particularly valuable for experimental sampling in human studies and in large-animal models [35]. While bronchoscopic sampling is designed to directly access the airways and alveoli, there remains a theoretical risk of pharyngeal contamination. To reach the lower respiratory tract (LRT), the bronchoscope must first pass through the pharynx and larynx, which exposes it to the upper respiratory tract (URT), where bacterial density is relatively high. This raises the possibility of inadvertently introducing pharyngeal bacteria into LRT samples. However, evidence from multiple

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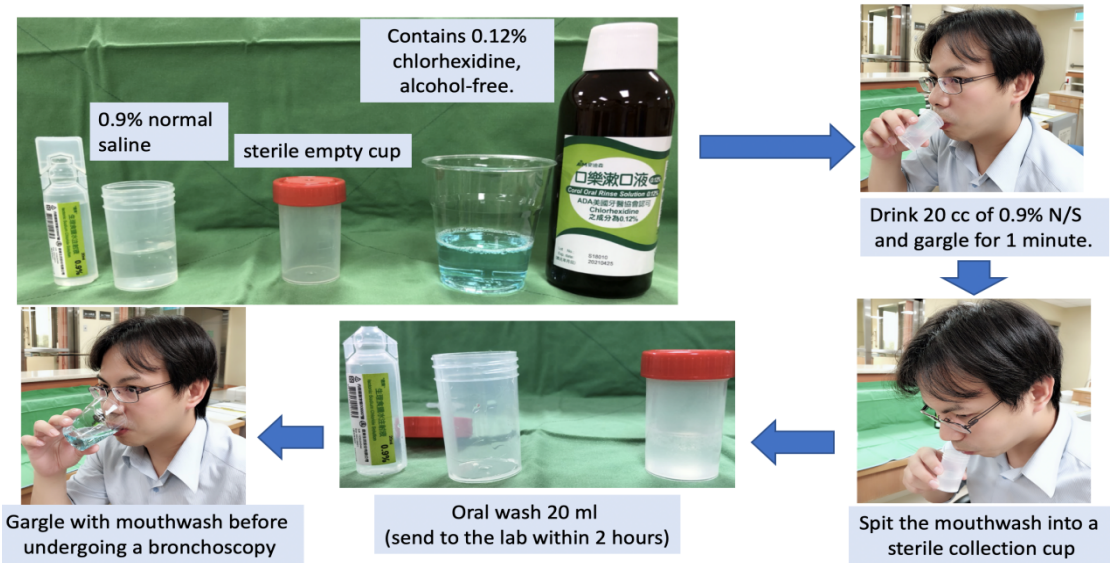
studies, both direct and indirect, suggests that pharyngeal contamination has minimal impact on the accuracy of bronchoscopically acquired specimens [35,39]. Bronchoalveolar lavage is a widely used, clinically established, and easily interpretable method for sampling the distal airways and alveolar space. During the procedure, the bronchoscope is advanced into a segmental or subsegmental airway until it is "wedged" in place. Once wedged, sterile saline is instilled into the airway and then suctioned out, collecting the lavage fluid for analysis [9,35]

The following is our BAL protocol for sampling lung microbiota in study participants (Figure 6-8).

1) Before bronchoscopy exam: oral wash fluid collection (Figure 6)

Figure 6. Before bronchoscopy exam: oral wash fluid collection (OWC control sample)



2) Before the procedure: 20 mL of sterile 0.9% saline was also washed through the

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bronchoscope and collected as a negative control sample (Figure 7)

Figure 7. Before the procedure: 20 mL of sterile 0.9% saline was also washed through the bronchoscope and collected as a negative control sample

Bronchoscope suction channel washing control (BWC)

Normal saline control 20 ml (NSC)

3) Bronchoalveolar lavage collection technique (Figure 8)

Figure 8. Bronchoalveolar Lavage (BAL) Collection Technique

Manual aspiration around 40-50% BAL fluid back and in general, BAL collection with BALF up to 40-60 ml.

(1). Patients should fast for 4 hours prior to undergoing bronchoscopy.

(2). A local anesthetic of 2% lidocaine (Xylocaine) is applied to the throat and airway

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mucosa.

(3). A fiberoptic bronchoscope is inserted through the mouth into the trachea and wedged into the bronchial opening at the lesion site. If there are no specific lesions, samples are typically collected from the right middle lobe (RML).

(4). Using a 25 ml syringe, saline is injected in portions, 25-50 ml at a time. After each injection, allow the patient to breathe for 5 cycles (25-30 seconds) before suctioning and recovering the lavage fluid into a silicone collection bottle.

(5). Manual aspiration if possible and gently bronchial suction and the expected recovery volume should be 40%-60% of the injected volume and in general with 40-60 ml BAL fluid retrieval.

(6). The ideal minimum total recovery volume of the bronchoalveolar lavage fluid is 50 ml. If the total recovered volume is less than 50 ml, additional saline will be injected in 25-50 ml increments until the maximum total saline volume reaches 200-250 ml [with at least 20 ml of BAL sample recovered].

One limitation of BAL is the variability in dilution: even when a fixed volume of saline is instilled, the amount of fluid recovered can vary significantly. However, this does not hinder the interpretation of lung microbiome data because: (1) relative abundance data

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is compositional, representing fractions of the whole rather than absolute values, and (2) the variation in bacterial DNA load across specimens (which can range from 100- to 1000-fold) far exceeds the relative differences in lavage volumes [40,41]

1.3.3 Contamination and negative control sample collection

An important consideration in lung microbiome studies is how to control for the high risk of contamination due to low biomass in samples [13, 42]. In lung microbiome studies, contamination is a critical issue, especially in low-biomass sequencing. Such studies face two significant sources of noise: reagent contamination and the inherent stochasticity of low-biomass sampling. Bacterial DNA is present in the environment and is not completely eliminated by conventional sterilization methods. This leads to the detection of bacterial taxa in laboratory reagents, which can introduce bias into microbiome studies in a kit-specific manner [42]. Contamination from reagents is a major source of Type I (false-positive) errors, especially in ultra-low-biomass studies like those focusing on the lung microbiome. Bacterial DNA from the environment or poorly sterilized equipment can skew results, making it appear as if microbial taxa are present in the sample when they may not be [35].

In addition to contamination, low-biomass (such as BAL sample) studies are vulnerable to the stochasticity of sampling sparse DNA populations. Just as small sample

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sizes can lead to unrepresentative survey results, sparse microbial communities may produce erratic sequencing outcomes. This noise can create results that are either "reproducibly wrong" (from contamination, where results are similar across samples) or "irreproducibly wrong" (from stochastic sampling, where results differ greatly between samples [30,35].

Figure 9. Signal and Noise in microbiome sampling (adopted from Reference 35)

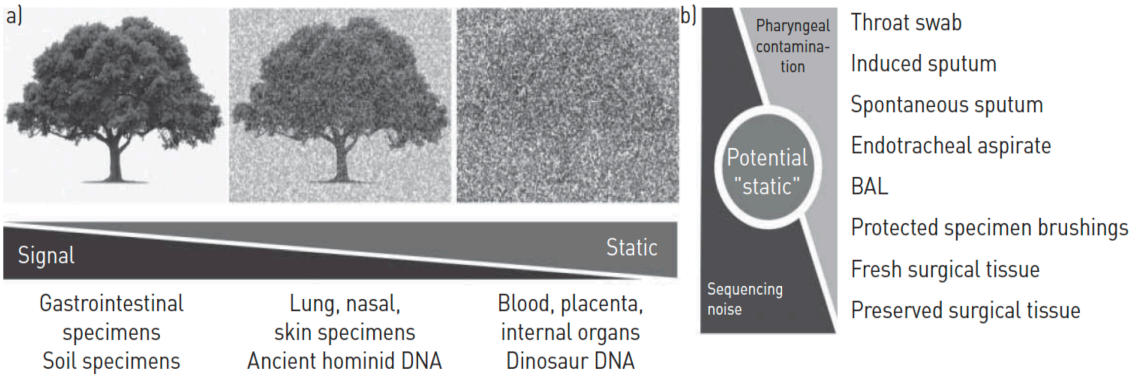

Figure 9 presents a conceptual framework for understanding the balance between "signal and static" in lung microbiome studies. This framework illustrates how samples differ across the **signal–static spectrum**. In microbe-rich samples, such as stool and soil, the strong bacterial signal easily overwhelms background noise from procedural or sequencing contamination. Conversely, in ultra-low-biomass samples like blood, placenta, and brain, contamination can dominate any genuine microbial signal [35].

Lung microbiome specimens, such as sputum and BAL samples, like those from other low-biomass sites such as the skin and nasal rinses, fall between these extremes.

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Special precautions are necessary to distinguish the true microbial signal from contamination-related noise. Additionally, respiratory specimens vary in their susceptibility to pharyngeal and sequencing contamination, which are primary sources of noise when detecting lung microbiome signals [13,35].

In our study, we initially chose to use BAL samples, which are considered low-biomass and, therefore, more susceptible to background contamination. To mitigate this, we also collected oral biological controls (oral washing fluid) and background negative controls (including bronchoscope channel washing fluid, sterile saline, and reagents) from the study participants (**Figures 6-10**). These control samples were utilized in our sequence analysis for the **decontam method** to help differentiate genuine microbial signals from contamination.

Figure 10. Negative control and biological control samples

- **Negative control for background contamination**
1. Bronchoscope suction channel washing control (BWC)
2. Normal saline control (NSC)
- **Negative control for reagents contamination**
1. Cell suspension (PBS) control
2. Extraction kit control
3. Non-templete control
- **Biological samples and control**
1. BAL samples (Lung microbiome)
2. Oral washing control (Oral microbiome)

Table 1 presents a comparison of the limitations of BAL samples and sputum samples. In our initial study, we aimed to evaluate the effectiveness of sampling in

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conditions such as COPD, bronchiectasis, and interstitial lung diseases (ILDs). We also considered the potential impact of therapeutic interventions that might reduce sputum production, thereby limiting the reliability of sputum samples. For these reasons, we opted to use BAL samples. Additionally, this approach was considered for use in animal studies [35].

Table 1 The Comparison of sputum sample and BAL samples

	Advantage	Disadvantage
Sputum sample	➢ Easy to collect, with low risk. ➢ Samples can be easily repeated. ➢ Higher microbial load. ➢ Suitable for severe patients who cannot undergo bronchoscopy.	➢Not every airway disease produces sputum. ➢Sputum induction may not be feasible for all patients. ➢Easily contaminated by saliva (quality of sputum is hard to control, introducing bias). ➢Sputum volume can be affected by treatment, making long-term follow-up difficult. ➢Sputum can be thick and hard to process (requiring the addition of dithiothreitol, e.g., Sputasol), which increases the risk of background contamination.
BAL sample	➢Currently considered the best sampling method for lung microbiome research in both humans and animals. ➢Truly reflects the microbiota of the lower respiratory tract (bronchial tree and alveolar space). ➢Minimal pharyngeal contamination. ➢BAL dilution variability does not interfere with lung microbiome data interpretation, because: Relative abundance is measured. ➢ Bacterial DNA load variation (100–1000-fold) is much greater than BAL dilution effects.	➢ Higher procedural risk, sometimes requiring sedation or anesthesia. ➢Not easily repeatable. ➢Not suitable for severely ill patients who cannot undergo bronchoscopy. ➢Low biomass specimens (requiring negative controls). ➢Risk of procedural contamination.

1.4 DNA extraction protocol

When extracting bacterial DNA from a sample, it's crucial to minimize bias, as bacteria have diverse cell wall properties that can complicate DNA extraction. Choosing the right extraction method helps avoid PCR inhibitors and reduces bias in the representation of bacterial taxa. Adding chemical and mechanical lysis steps can improve yields, especially for Gram-positive bacteria. While DNA extraction kits are

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convenient, they may introduce contamination, so using extraction controls is essential.

Figure 11A (information adopted from website)

Kit Contents

QIAamp DNA Microbiome Kit	**(50)**
Catalog no.	**51704**
Number of preps	**50**
QIAamp UCP Mini Columns	50
Collection tubes (2 ml)	150
Pathogen Lysis tubes L	50
Elution tubes (1.5 ml)	100
Buffer AHL	5 bottles
Buffer RDD	35 ml
Benzonase®	2 vials
Buffer ATL	50 ml
Reagent DX (clear cap)	1 ml
Buffer APL2*	14 ml
Buffer AW1* (concentrate)	19 ml
Buffer AW2† (concentrate)	13 ml
Proteinase K (green cap)	2 vials
Buffer AVE†	3 vials
Quick-Start Protocol	1

* Contains chaotropic salt. Not compatible with disinfecting agents containing bleach.
† Contains sodium azide as a preservative.

In our study, we used the QIAamp DNA Microbiome Kit [43] **(Figure 11A)** to effectively remove host DNA from BAL fluid samples. This kit is specifically designed for low-biomass microbiome samples, helping isolate microbial DNA while minimizing contamination from host cells. By targeting and depleting human DNA, the kit ensures that downstream sequencing focuses on the microbial component, enhancing the accuracy of lung microbiome analysis. This step was crucial for our study, as BAL fluid contains a significant amount of host DNA that could otherwise interfere with the detection of the microbial population.

The following protocol (**Figures 11B-11C**) outlines the DNA extraction procedure for

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the BAL pellet used in our study. It includes steps like host cell lysis using ATL buffer and proteinase K, followed by microbial DNA isolation to minimize host DNA contamination. This ensures high-quality microbial DNA for accurate lung microbiome analysis [43].

Figure 11B. The process of DNA extraction for BAL pellet (I)

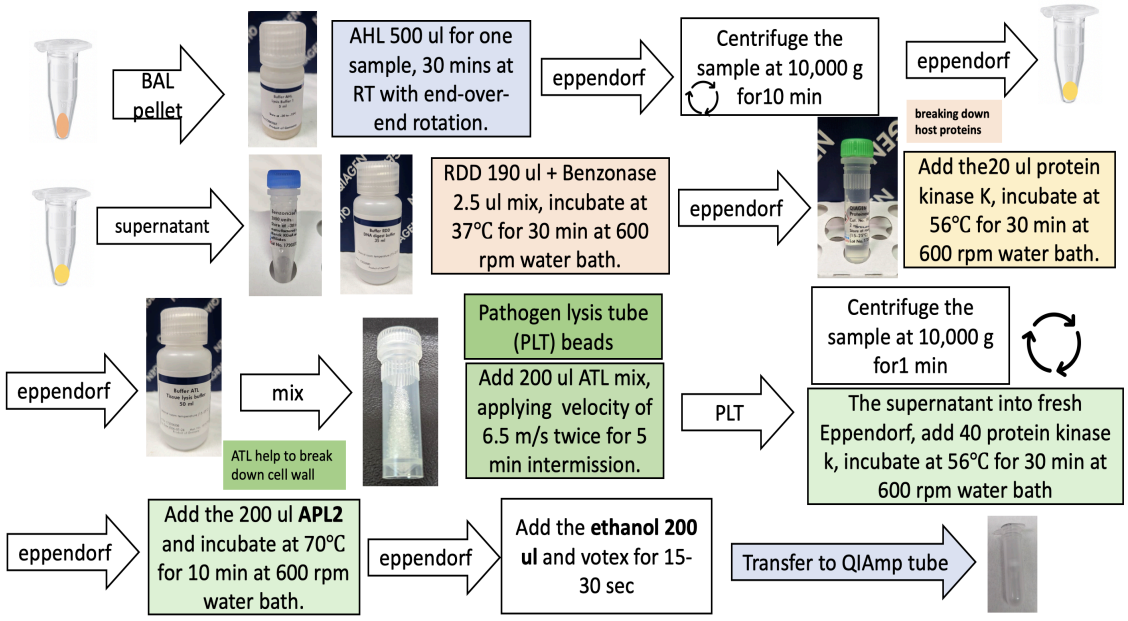
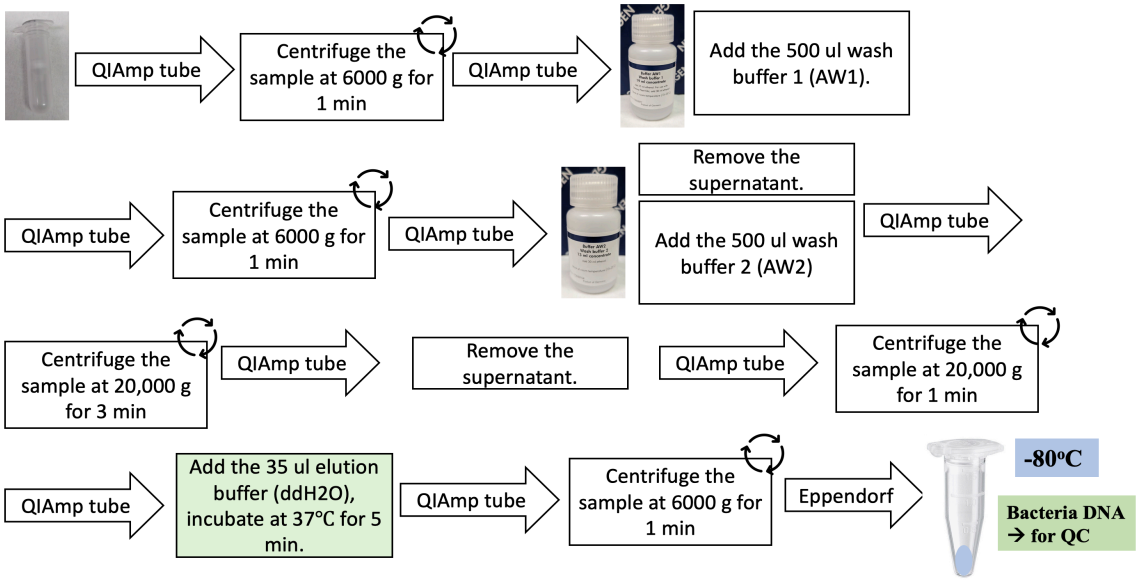


Figure 11C. The process of DNA extraction for BAL pellet (II)



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1.5 From Library preparation, Cluster generation, Sequencing to Alignment

In essence, the principle behind NGS technology is similar to Sanger sequencing, also known as capillary electrophoresis (CE) sequencing, where DNA polymerase catalyzes the incorporation of fluorescently labeled deoxyribonucleotide triphosphates (dNTPs) into a growing DNA strand during each cycle of DNA synthesis. During each cycle, the incorporated nucleotide is identified through fluorophore excitation. The key distinction is that NGS scales this process to millions of DNA fragments simultaneously, enabling massively parallel sequencing. Today, more than 90% of the world's sequencing data is produced using Illumina's sequencing by synthesis (SBS) technology, which provides high accuracy, a large output of error-free reads, and a high percentage of base calls with quality scores above Q30 [44,45,46,47].

1.5.1 Library preparation:

This step prepares DNA samples to make them compatible with the sequencing platform [44].

(1) Fragmentation: The DNA is broken down into manageable sizes (typically 200-500 base pairs) using techniques such as sonication or enzymatic digestion.

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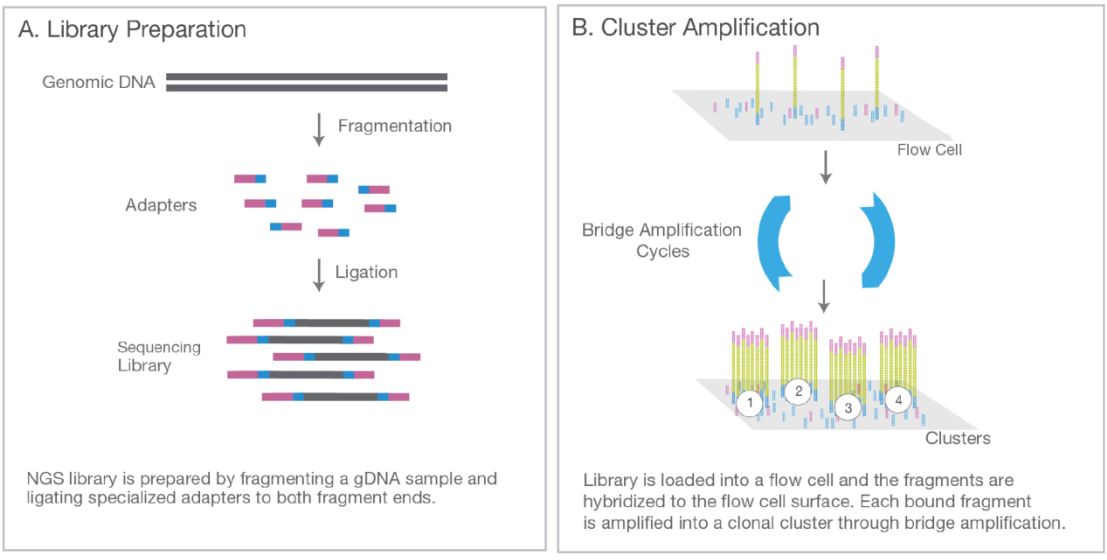
(2) **Adapter ligation:** Short sequences, known as adapters, are ligated to both ends of the fragmented DNA. These adapters contain sequences required for binding primers, identifying samples (using indexes), and facilitating attachment to the sequencing flow cell (**Figure 12A**).

(3) **Tagmentation:** In some protocols, fragmentation and adapter ligation are combined into a single step, making the process more efficient. This combination is referred to as tagmentation [48].

(4) **Enrichment:** Through PCR amplification, only the DNA fragments containing adapters are amplified, ensuring that only target sequences are sequenced

Figure 12. Overview of the (A) Library Preparation and (B) Cluster Amplification processes (Adapted from Reference 44)



1.5.2 Cluster amplification:

After library preparation, the DNA fragments are introduced to the flow cell for cluster generation.

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(1) Flow cell attachment: The flow cell contains oligonucleotides that are complementary to the adapter sequences. The DNA fragments attach to these oligonucleotides on the flow cell.

(2) Bridge amplification: The attached DNA fragments bend to form "bridges" with adjacent oligonucleotides, allowing them to be copied multiple times via bridge amplification. This results in the formation of clusters, with each cluster comprising thousands of identical copies of the original DNA fragment.

Importance: Cluster generation enhances the signal for sequencing, ensuring that the system can accurately detect the DNA sequences. (**Figure 12B**)

1.5.3 Sequencing:

Once clusters are generated, the sequencing process—Sequencing by Synthesis (SBS)—begins **(Figure 13A)**

(1) Nucleotide incorporation: Fluorescently labeled nucleotides (A, T, C, G) are introduced to the system. Each type of nucleotide has a unique fluorescent tag. Illumina's SBS technology utilizes a proprietary reversible terminator-based method that detects single bases as they are incorporated into DNA template strands.

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(2) Fluorescence detection: During each sequencing cycle, all four types of reversible terminator-bound dNTPs are present, allowing natural competition between nucleotides. This minimizes incorporation bias and significantly reduces raw error rates compared to other sequencing technologies. As each nucleotide is added to the growing DNA strand, the emitted fluorescence is captured by a camera, identifying the nucleotide that was incorporated.

(3) Cycle repetition: The process is repeated in cycles, with one nucleotide being added per cycle. The cycles continue until the entire DNA fragment is sequenced. Millions of clusters are sequenced in parallel, producing large amounts of sequencing data. Due to the highly accurate base-by-base sequencing, this method virtually eliminates sequence context-specific errors, even in challenging regions like repetitive sequences and homopolymers.

1.5.4 Alignment and Data analysis

Once sequencing is complete, the reads are aligned for further analysis **(Figure 13B) [44]**.

(1) **Data processing:** The sequencer generates base sequences from each cluster, which are then aligned to a reference genome (see **Figure 4D**). After alignment, various analyses are possible, including single nucleotide polymorphism (SNP) or insertion-

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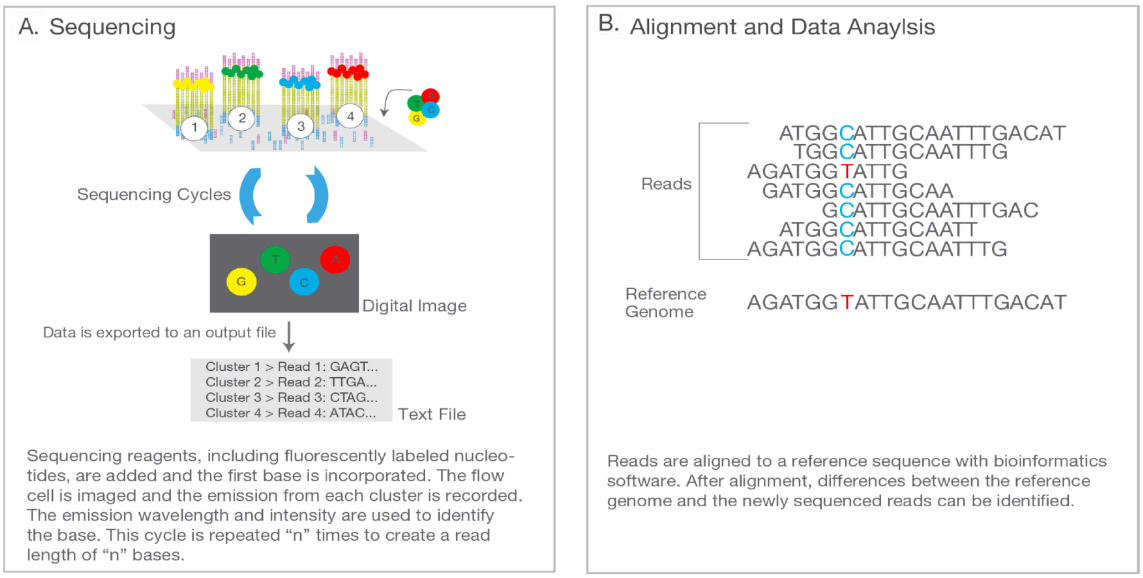
deletion (indel) identification, RNA read counting, and phylogenetic or metagenomic analysis.

(2) **Error correction:** During alignment, computational tools are employed to correct sequencing errors or mismatches between the reads and the reference genome.

(3) **Read assembly:** In the case of de novo sequencing (where no reference genome is available), overlapping reads are assembled to form longer contiguous sequences, allowing researchers to reconstruct the genome from scratch.

Figure 13. Overview of the (A) Sequencing and (B) Alignment and Data analysis (Adapted from Reference 44)



1.6 Sequence analysis

Microbiome data can reveal different aspects of a bacterial community. 16S rRNA gene sequencing typically provides information on the community's composition, though resolution is generally limited to the genus level. In contrast, shotgun

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metagenomics (DNA sequencing) and metatranscriptomics (RNA sequencing) offer insights into both composition (at the species or strain level) and functional potential or activity. Regardless of the platform or sequencing method, raw sequence data must first be processed to generate microbiota profiles—determining which bacteria are present and their relative abundances—before moving on to downstream analyses [7].

Several well-established bioinformatics pipelines, such as **mothur and QIIME (Quantitative Insights Into Microbial Ecology)** [7,49,50], handle these preprocessing steps. While the open-source nature of these tools facilitates analysis, it is important to be mindful of the nuances in parameter choices, such as similarity thresholds, reference database selection, and the algorithm used for defining operational taxonomic units (OTUs) or amplicon sequence variants (ASVs), as these can significantly impact results (**Figure 14 and Figure 15**)

1.6.1 Operational Taxonomic Units (OTUs) versus Amplicon Sequence Variants (ASVs)

The difference between Operational Taxonomic Units (OTUs) and Amplicon Sequence Variants (ASVs) lies in how they define and categorize sequences from microbiome data [7]

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(1) OTUs (Operational Taxonomic Units):

OTUs are clusters of sequences that are grouped together based on a certain percentage of similarity, traditionally 97%. This means that sequences within an OTU are at least 97% identical, and the cluster represents a proxy for a bacterial species. OTUs are used as a molecular approximation of species when using 16S rRNA gene sequencing. Since it is difficult to define bacterial species, OTUs serve as a practical unit of comparison. The method of clustering sequences into OTUs can vary, and there is no single optimal way to do this, which has led to ongoing debates in the scientific community regarding the most accurate clustering thresholds (e.g., 97%, 99%, or 100%) [7, 51] (**Figure 14**)

(2) ASVs (Amplicon Sequence Variants):

ASVs represent exact sequences after error correction, without clustering by percentage identity. Unlike OTUs, which lump similar sequences together, ASVs keep individual sequence variants separate [7,51]. ASVs aim for higher resolution by removing errors from the sequence data and treating each unique sequence as a representation of an organism in the sample. ASVs have gained popularity due to their increased precision in representing true biological diversity and are not dependent on arbitrary thresholds like OTUs. Pipelines such as DADA2 [52], UNOISE2 [53], and Deblur [54] are commonly

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used to process sequence data and identify ASVs through different error-removal algorithms [51,52,55,56] (**Figure 15**)

Comparison of Operational Taxonomic Units (OTUs, Figure 14) and Amplicon Sequence Variants (ASVs, Figure 15) (Adapted and modified from Reference 56).

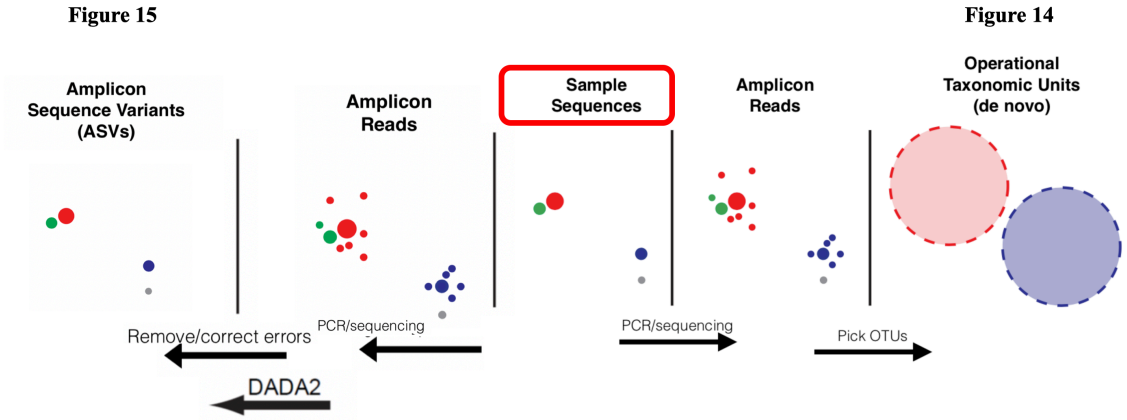

Figure 14. OTUs (Operational Taxonomic Units) are traditionally constructed using clustering methods, often based on 97% or 99% sequence similarity thresholds. While OTU clustering has been widely used for microbial analysis, it is acknowledged that OTUs do not perfectly represent individual species, thus limiting the accuracy of species-level analysis. In recent years, many have advocated for moving away from the less precise OTU classification in favor of denoising methods that generate higher-resolution **ASVs (Amplicon Sequence Variants. Figure 15.)** One such method is DADA 2(Divisive Amplicon Denoising Algorithm 2), which corrects sequencing errors in amplicon data without relying on clustering to construct OTUs. Instead, it directly analyzes sequence variations, providing greater resolution and accuracy in representing true biological diversity [55].

1.7 The origins of the lung microbiota

The lung microbiota is shaped by a conceptual ecological model balancing three key factors: immigration, elimination, and regional growth factors, all influenced by environmental conditions [57] **(Figure 16)**. In healthy lungs, immigration primarily

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occurs through microaspiration of pharyngeal contents [58], while elimination is driven by coughing, mucociliary clearance, and host defenses. Environmental factors such as temperature, pH, and oxygen tension vary within the lungs and across disease states.

Figure 16: The ecologic determinants of the lung microbiome (Adapted from Reference 57).

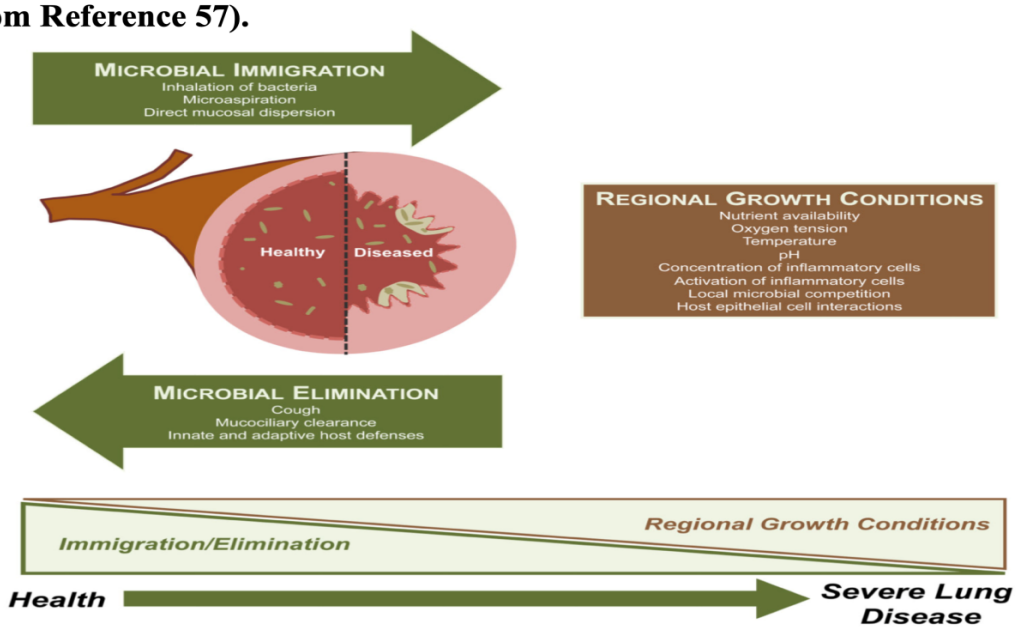

Figure 16: The ecological factors shaping the lung microbiome are influenced by microbial immigration, elimination, and changes in microbial growth based on environmental conditions. In a healthy lung, minimal microbial reproduction occurs, and the lung microbiome is primarily maintained through a balance of immigration (e.g., microaspiration) and elimination. However, in severe lung disease, altered environmental conditions—such as increased nutrient availability—lead to the development of distinct, disease-specific microbial communities adapted to the conditions of damaged airways (adapted from Reference 57).

In this model, the lungs act as islands influenced by microbial immigration from the upper respiratory tract (URT) or ambient air and elimination through host defenses. The balance between immigration and extinction determines bacterial richness in the lungs. Regional growth factors, such as proton-pump inhibitors, laryngeal function, increased

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microbial burden, gastrointestinal reflux, and hyperventilation, can alter this balance. Extinction rates depend on factors like coughing frequency, ciliary clearance, airway obstruction, and immune responses. Shifts in immigration or elimination rates can affect species richness, with potential clinical implications [57].

In severe lung disease, altered environmental conditions, such as increased nutrient availability, drive the formation of disease- and patient-specific microbial communities, optimized for the injured airways and reflecting the microbiota's adaptation to new ecological pressures [5,57,58].

1.8 The concept of "bacterial dysbiosis" in chronic lung disease

In healthy individuals, the airway microbiota is diverse and well-balanced, contributing to normal respiratory function. "**Dysbiosis"**, an imbalance in the microbial community, occurs in chronic lung disorders such as asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis, and bronchiectasis [5,59,60]. This imbalance often results from the overgrowth of certain bacterial species, leading to a disruption in the normal microbial diversity (**Figure 17**).

Patients with asthma and COPD exhibit notable similarities in the bacteria associated with dysbiosis [60]. In asthma, dysbiosis is characterized by an overgrowth of the *Proteobacteria* phylum and a shift in the proportion of *Streptococci* within the *Firmicutes*

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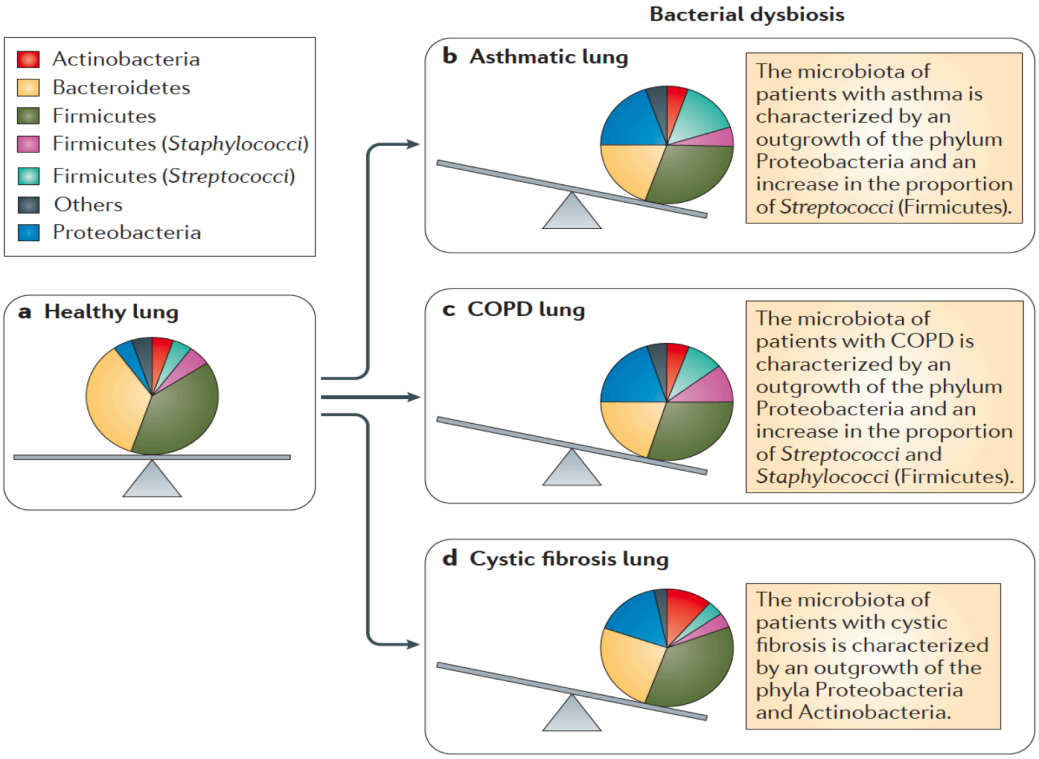
phylum. Similarly, in COPD, there is an increase in such as *Staphylococci Streptococci* and *Haemophilus,* alongside a significant expansion of *Proteobacteria* [61,62].

Figure 17: Bacterial dysbiosis in chronic lung disorders (adapted from Reference 5)



In cystic fibrosis (CF), the nature of bacterial shifts differs slightly. As in asthma and COPD, *Proteobacteria* dominate the dysbiotic community, but unlike these conditions, no major changes are observed in the *Firmicutes* phylum. Instead, *Actinobacteria* are significantly overrepresented in CF patients, indicating a unique microbial imbalance in this disease [5,60]. In bronchiectasis, dysbiosis presents as a decrease in microbial diversity, often with an overgrowth of *Pseudomonas aeruginosa* and other members of the *Proteobacteria* phylum [59]. Similar to cystic fibrosis, bronchiectasis is characterized by chronic infection and inflammation, which further promote the overgrowth of

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pathogenic species while diminishing the presence of commensal bacteria. This microbial imbalance exacerbates the progression of airway damage and contributes to the cycle of infection and inflammation commonly seen in bronchiectasis patients [63]. Dysbiosis in each of these conditions can further exacerbate disease progression by fostering a pathogenic environment in the airways.

1.9 The role of lung microbiome in chronic lung diseases (COPD and bronchiectasis)

Research has shown that the lung microbiota is not only detectable in healthy individuals but also undergoes significant alterations in various lung disease states [3,4,5], including COPD [64,65,66] and bronchiectasis [67,68,69]. In both of these conditions, the microbial diversity in the lungs is often reduced, and pathogenic bacteria may dominate, contributing to chronic inflammation and disease progression [64,65,66,69]. Variations in microbial diversity and composition have been linked to different health outcomes, and shifts in these communities have been associated with changes in host immune responses. This growing body of evidence suggests that the lung microbiome plays a critical role in maintaining respiratory health and in the pathogenesis of these conditions [66,69].

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1.10 The association of COPD and bronchiectasis

Patients with bronchiectasis and fixed airflow obstruction (FAO) are those who meet both the obstructive spirometry criteria for COPD and the structural diagnosis of bronchiectasis [70,71]. The clinical and pathological features coexisting in bronchiectasis and COPD can exacerbate symptoms, intensify inflammation, and worsen prognosis compared to either condition alone [70-75]. A new consensus regarding the definition of "COPD–bronchiectasis association" was proposed by the EMBARC Airway Working Group recently [76]; this definition comprises four components, namely specific radiological signs, functional obstructive pattern, at least two characteristic respiratory symptoms, and current or past smoking (≥ 10 pack-years) or biomass exposure (i.e., ROSE criteria), which are used to describe the coexistence of these two disease entities with complex interactions.

1.11 The lung microbiome in COPD-BE association?

Dysbiosis in the lung microbiome, particularly involving *Proteobacteria* such as *Pseudomonas* and *Haemophilus*, is linked to increased severity and exacerbations in COPD [64,65,66,74] and bronchiectasis patients [67,68,77]. However, the role of the lung microbiome in bronchiectasis patients with fixed airflow obstruction or so-called "bronchiectasis and COPD overlap" remains an under-researched area [74,78]. A recent study [75] analyzing a United Kingdom cohort used sputum samples to identify five

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endotypes, revealing distinct inflammatory statuses and microbiological characteristics in COPD, bronchiectasis, and the "COPD-bronchiectasis association" as per the ROSE criteria [76].

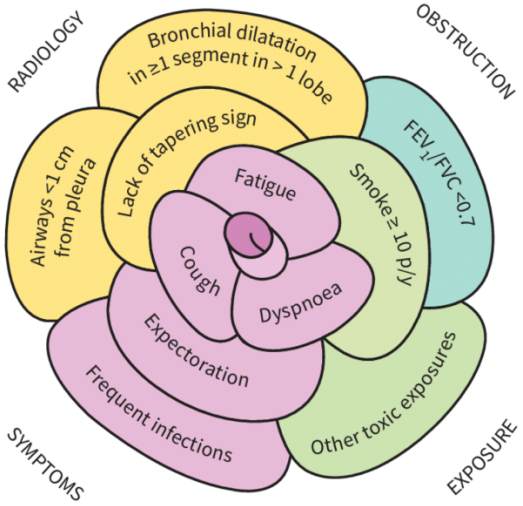

Figure 18 ROSE Criteria1:

1.RADIOLOGICAL:Abnormal bronchial dilatation in one or more pulmonary segment in more than one lobe and specific radiological findings (airways visible within 1 cm of pleura and/or lack of tapering sign) plus

2.OBSTRUCTION:A functional obstructive pattern (post-bronchodilator FEV1/FVC <0.7), plus

3.SYMPTOMS: two or more of the following symptoms: cough, expectoration, dyspnea, fatigue, frequent lower airway infections (⩾2/year) plus

4. EXPOSURE: current or past smoking habit (⩾10 pack-years) or other toxic exposure (biomass, industrial, etc.)

Figure 18 adapted from Reference 76)

This research underscored that traits like neutrophilic inflammation, differential mucin expression, and gram-negative infections are prevalent in patients with the "COPD-bronchiectasis association". Nevertheless, there is a notable gap in robust data for advanced bronchiectasis patients with fixed airflow obstruction (FAO) and typical airway symptoms [71], particularly those who do not meet the ROSE criteria due to a lack of smoking history. Additionally, regional variances in etiology, smoking patterns, and environmental exposures in East Asia and other areas may uniquely affect lung microbiology in both COPD [79,80,81] and bronchiectasis [82,83]. The complex interplay between the lung microbiome, smoking exposure, and bronchiectasis with

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airflow obstruction [71,72,75,78,82] is increasingly recognized. Yet, there is a scarcity of research specifically addressing these relationships within East Asian populations.

1.12 Aim of study for Lung microbiome research for COPD and bronchiectasis

In this study, our objective is to investigate the role of lung microbiome in COPD and bronchiectasis patients using bronchoalveolar lavage (BAL) samples. We also aim to evaluate airway inflammatory markers and their clinical relevance, categorizing these patients based on their adherence to the ROSE criteria [76]. Additionally, we will compare these findings with those from patients diagnosed solely with COPD or bronchiectasis within an East Asian cohort.

1.12.1 Establish a prospective clinical study for COPD

Design a longitudinal study to collect BAL samples from COPD patients and monitor disease progression, exacerbations, and treatment responses, while also investigating the lung microbiome. Baseline data, including spirometry, imaging, biomarkers, and microbiome composition, will be gathered to provide a comprehensive understanding of COPD and its relationship with microbial changes in the airways. To establish a prospective clinical study for Bronchiectasis

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1.12.2 Establish a prospective clinical study for bronchiectasis

Create a prospective study to collect BAL and stoll samples from bronchiectasis patients, tracking disease progression, exacerbations, and treatment effects (such as antibiotics). Baseline lung function and microbiome assessments will help identify key influencing factors.

1.12.3 Investigate the lung microbiome in COPD, Bronchiectasis, and coexisting conditions using BAL samples

Analyze the lung microbiome in COPD, bronchiectasis, and coexisting conditions through BAL samples, focusing on microbial composition, dysbiosis, and disease severity, and how these factors are associated with clinical outcomes. To apply the ROSE criteria to assess the prevalence, clinical impact, and outcomes of the COPD-BE association in an East Asian cohort

1.12.4 Apply the ROSE criteria to assess the COPD-Bronchiectasis association in East Asian cohorts

Apply the ROSE criteria to assess the prevalence, clinical impact, and outcomes of coexisting COPD and bronchiectasis in an East Asian population, focusing on the effect on lung function, exacerbation rates, and mortality.

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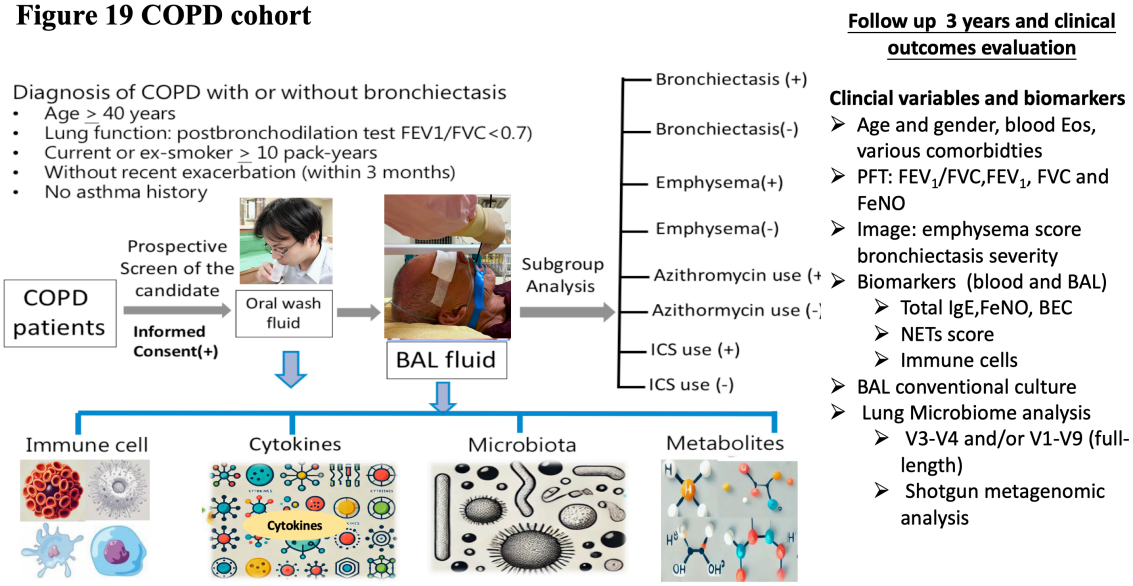
Chapter 2. Materials and Methods

2.1 Study design and participants

Patients with a clinical diagnosis of bronchiectasis or COPD were prospectively recruited between November 2018 and February 2022 from the National Taiwan University Hospital (NTUH), Yunlin branch, Yunlin County, Taiwan. We recruited clinically stable patients diagnosed with COPD **(Figure 19**) and Bronchiectasis **(Figure 20**) according to the relevant guidelines [84,85,86].

Figure 19 COPD cohort



2.2 Inclusion criteria

Patients were enrolled if they were aged ≥ 40 years, had a forced expiratory volume in one second (FEV_1)/forced vital capacity (FVC) ratio < 0.7 at a screening visit, and had a smoking history of at least 10 pack-years or relevant biomass exposure [84]. Bronchiectasis was confirmed by a high-resolution computed tomography (HRCT) scan

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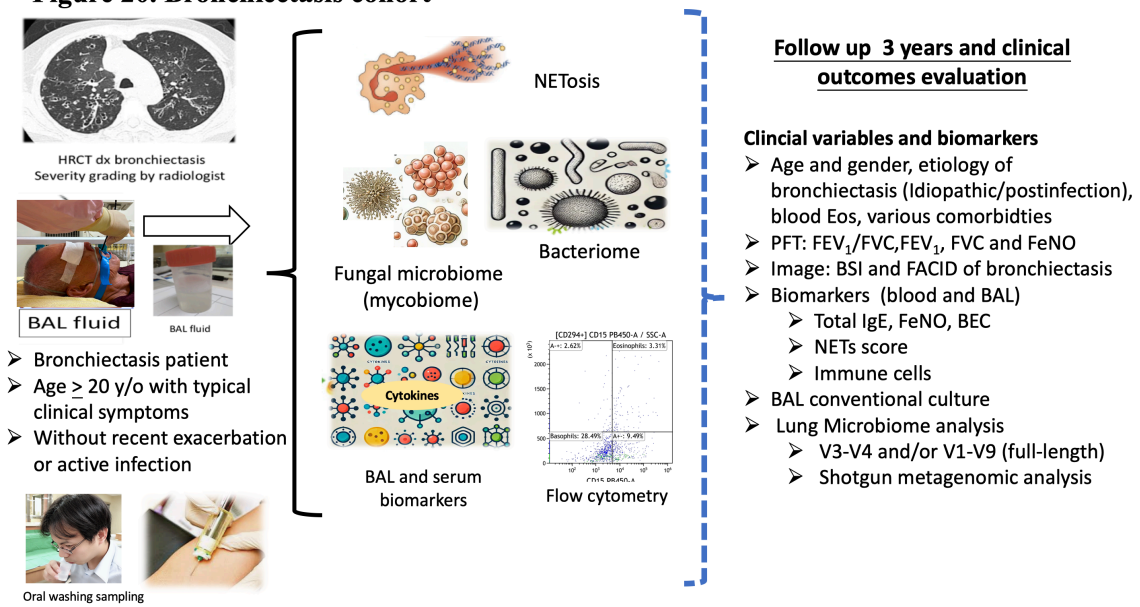
indicating a bronchoarterial ratio > 1, lack of tapering, and airway visibility within 1 cm of the pleural surface [85,86] (**Figure 20**), along with clinical symptoms consistent with bronchiectasis. The definition of bronchiectasis with FAO was based on broadly established criteria, encompassing typical airway symptoms (e.g., cough, shortness of breath, wheezing, and sputum production) that met both spirometry criteria for COPD and the structural diagnosis of bronchiectasis [71].

Figure 20. Bronchiectasis cohort



2.3 Exclusion criteria

Patients were excluded if they

(1) Patients had cystic fibrosis-related bronchiectasis, active allergic bronchopulmonary aspergillosis (ABPA), active pulmonary tuberculosis, or a current diagnosis of asthma.

(2) Patients had acute exacerbation of COPD or bronchiectasis within the past 3

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months

(3) Currently under specific antibiotic treatments or had an acute infection within 1 month before the study with or without antibiotic exposure

(4) Patients on long-term antibiotics or undergoing chemotherapy for malignancy were also excluded.

2.4. Clinical assessment of the patients

We collected comprehensive clinical data at enrollment, including demographics, clinical manifestations, comorbidities, laboratory data, microbiology, lung function, imaging studies, clinical outcomes, history of exacerbations, current inhalation medications, and past major conditions and differential diagnoses were meticulously documented by experienced clinicians to ensure accuracy and reliability.

2.4.1 Lung function measurements

Lung function testing was conducted at baseline in accordance with the technical standards set by the ERS and the American Thoracic Society (ATS) [87]. The percentage of predicted FEV_1 was calculated using reference values established for the Taiwanese cohort [88]. Airflow obstruction in bronchiectasis was defined by a post-bronchodilator FEV_1/forced vital capacity (FVC) ratio of less than 0.7 [89], following the ROSE criteria [76] and supported by previously published reports [90,91]

2.4.2 Quantification of emphysema area on chest CT

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All study participants underwent a CT quantification to assess the severity of emphysema. The emphysema severity was quantified by measuring the low-attenuation volume (LAV %), which was segmented at a threshold of -930 Hounsfield units (HUs) relative to the total lung volume on inspiratory CT images [92,93].

2.4.3 Assessment of severity of bronchiectasis

For bronchiectasis, radiological severity of bronchiectasis was quantified using a modified Reiff score, with a range from 1 (minimum) to 18 (maximum) for lobar involvement assessment [94]. Additionally, the multidimensional bronchiectasis severity index (BSI) (**Table 2**), with clinical variables such as Age, BMI, FEV_1% Predicted, Hospital admission before the study, Exacerbation before the study, MRC

Table 2. Bronchiectasis severity Index (BSI) (adapted from Reference 95)

Severity Marker	HR (95% CI) for Hospital Admissions during Follow-up	HR (95% CI) for Mortality	Score Points
Age, yr			
<50	1.0 (reference)	1.0 (reference)	0
50–69	1.38 (0.73–2.56)	2.21 (0.28–17.5)	2
70–79	1.50 (0.79–2.82)	8.57 (1.15–63.63)	4
80+	1.76 (0.89–3.50)	23.16 (3.09–173.7)	6
BMI			
<18.5	1.23 (0.73–2.08)	2.25 (1.09–4.67)	2
18.5–25	1.0 (reference)	1.0 (reference)	0
26–29	0.90 (0.62–1.30)	0.91 (0.46–1.81)	0
30 or more	1.14 (0.76–1.70)	1.38 (0.68–2.81)	0
FEV_1 % predicted			
>80	1.0 (reference)	1.0 (reference)	0
50–80	1.17 (0.74–1.85)	1.34 (0.67–2.67)	1
30–49	1.40 (0.68–2.85)	1.58 (0.72–3.46)	2
<30	1.52 (1.03–2.25)	4.47 (1.60–12.53)	3
Hospital admission before study			
No	1.0 (reference)	1.0 (reference)	0
Yes	13.5 (9.40–19.46)	2.43 (1.30–4.53)	5
Exacerbations before the study			
0	1.0 (reference)	1.0 (reference)	0
1–2	1.67 (0.78–3.58)	1.78 (0.80–3.98)	0
3 or more	2.25 (0.89–5.70)	2.03 (1.02–4.03)	2
MRC dyspnea score			
1–3	1.0 (reference)	1.0 (reference)	0
4	2.42 (1.66–3.52)	1.05 (0.50–2.20)	2
5	2.69 (1.59–4.53)	1.15 (0.50–2.63)	3
Pseudomonas colonization			
No	1.0 (reference)	1.0 (reference)	0
Yes	2.16 (1.36–3.43)	1.58 (0.75–3.34)	3
Colonization with other organisms			
No	1.0 (reference)	1.0 (reference)	0
Yes	1.66 (1.12–2.44)	1.10 (0.54–2.24)	1
Radiological severity: ⩾3 lobes involved or cystic bronchiectasis			
No	1.0 (reference)	1.0 (reference)	0
Yes	1.48 (1.02–2.15)	1.05 (0.57–1.94)	1

Definition of abbreviations: BMI = body mass index; CI = confidence interval; HR = hazard ratio; MRC = Medical Research Council.
All factors founded to be significantly associated with either mortality or hospital admissions were included in the derivation of the severity score.

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dyspnea score, *Pseudomonas* colonization, Colonization with organisms and Radiological severity, which classifies bronchiectasis as mild (0–4), moderate (5–8), or severe (≥9), was also evaluated [95] (**Table 2**). Chest HRCT scans were reviewed by a trained chest specialist and a thoracic radiologist, both of whom were blinded to the clinical data. Moreover, E-FACED score, encompassing exacerbation, forced expiratory volume in 1 second (FEV_1), age, chronic colonization, disease extension, and dyspnea [96], was also utilized to comprehensively define bronchiectasis severity.

2.4.4. Clinical outcomes measurements

The definition of exacerbations in COPD and bronchiectasis was based on established guidelines. For COPD, according to the GOLD guidelines [84], a moderate exacerbation requires treatment with antibiotics or systemic glucocorticoids, while a severe exacerbation results in hospitalization or death. In bronchiectasis, an exacerbation was defined as at least three of the following symptoms persisting for ≥48 hours: worsened cough and sputum production, increased sputum purulence, breathlessness, new fever, fatigue, malaise, hemoptysis, Additionally, a necessary change in treatment for these symptoms is determined by a clinician based on the clinical assessment [97,98]. The severity of exacerbations for both conditions is graded according to the treatment required. Moderate exacerbation episodes necessitate outpatient treatment with antibiotics, systemic glucocorticoids, or other appropriate therapies [84,99], whereas

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severe exacerbation episodes require hospitalization or an emergency department visit due to airway complications [84,99]. The NTUH Research Ethics Committee approved the study (NTUH-REC No. 201712075RINA and 201910082RINA).

2.5 Sample collections and processing

2.5.1 BAL samples collections (see Figure 6-8)

The participants were asked to fast at least 4 hours before undergoing the BAL collection procedure. Participants gargled 20 mL of sterile 0.9% saline (for the collection of oral washing control samples) and then an antiseptic mouthwash containing 0.12% chlorhexidine gluconate immediately before undergoing topical anesthesia and conscious sedation. Before the procedure, 20 mL of sterile 0.9% saline was also washed through the bronchoscope and collected as a control sample. The bronchoscope was inserted into the mouth of a participant and quickly advance d to a wedge position.

In general, with up to 200 mL of 0.9% saline used, BAL fluid was predominantly collected from the right middle lobe in patients with COPD alone in accordance with published protocols [9]. For those with bronchiectasis, BAL fluid was preferentially collected from either the right middle lobe or the left lingual lobe based on the extent of the lobe involvement in bronchiectasis. BAL fluid collection was specifically targeted to the specific lobes with pronounced bronchiectatic changes. If similar levels of severity were noted in multiple lobes, BAL fluid was predominantly collected from the right

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middle lobe or the left lingual lobe. Although the most affected lobe may not always be the site of sample collection and variability in sampling locations may affect microbiome profiles, we ensured that the selected sites were clinically significant and indicative of active disease. This strategy allowed us to maintain the robustness of our findings while ensuring representative sampling, site accessibility, and patient safety. After the procedure, all the collected samples were sent to our lab within 2 hours for subsequent analysis.

2.5.2 BAL sample for cytokines analysis

The BAL supernatant was examined for various inflammation markers (e.g., tumor necrosis factor [TNF]-α, interleukin [IL]-1β, IL-6, IL-8, and IL-18) by using a ProcartaPlex Multiplex Immunoassays Kit (Thermo Fisher Scientific) to perform quantitative, multiplexed protein measurements and using Luminex magnetic bead technology per manufacturer recommendations [100].

2.5.3 BAL sample for immune cells analysis

The collected BAL fluid was filtered through a 40-μm cell strainer (Millipore, Billerica, discarded, and the conidial pellets were resuspended in 200 μL of phosphate-buffered saline (PBS) with the following monoclonal antibodies: CD14, CD15, CD16, CD45, CD49d, CD80, CD206, CD294 (Beckman Coulter), CD163, and CD193 (BioLegend, San Diego, CA, USA). The samples were stained at room temperature in the

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dark for 30 min and centrifuged at 200 × *g* for 5 min. Thereafter, the samples were resuspended in 400 μL of PBS/fix solution (1:1), and a flow cytometric assay (Beckman Coulter) was performed to assess their surface antigen levels [101].

2.5.4 BAL sample for neutrophilic extracellular traps (NETs) analysis

A 96-well plate was coated with myeloperoxidase (MPO) antibodies (1:500) with coating buffer and left overnight at 4°C. In each well, we replaced the coating buffer with 100 μL of incubation buffer at room temperature for 30 min. Next, in each well, we replaced the incubation buffer with 100 μL of sample buffer at 4°C, and this condition was maintained overnight. The wells were washed thrice with 300 μL of wash buffer. We added 100 μL of conjugate buffer for neutrophil's DNA dictation to each well at room temperature for 90 min and then washed each well thrice with 300 μL of wash buffer. Finally, we added 100 μL of substrate buffer at room temperature for 10–20 min and then used an enzyme-linked immunosorbent assay reader for analysis [102].

2.5.5 BAL sample processing for DNA extraction

A total of 10 mL of BAL fluid was centrifuged at high speed (13,000 rpm) to pellet cellular material. The bacteria genomic DNA in the BAL samples were extracted using a QIAamp DNA BAL kit (QIAamp DNA Microbiome Kit Cat. No./ID: 51704) according to the manufacturer's instructions (**Figure 11,12**) [43].

2.6 Methods for lung microbiome sequencing

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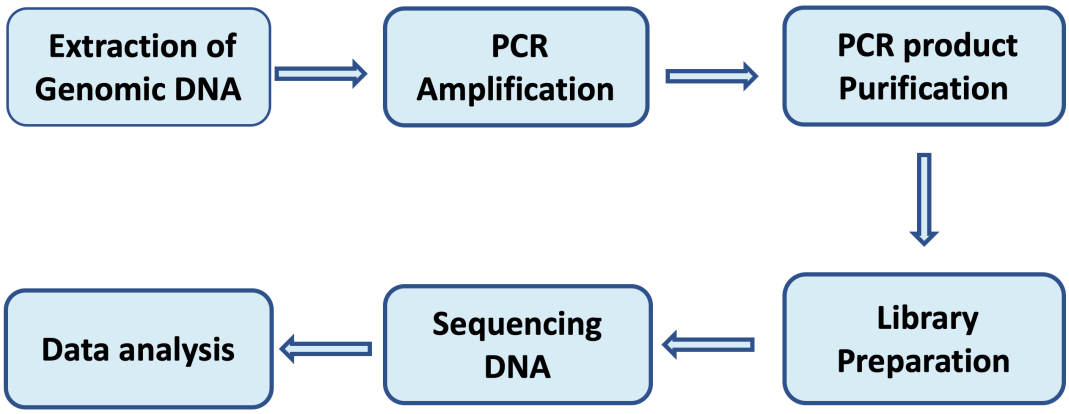
The pellets of human BAL fluid will apply for microbiota analysis as following (**Figure 21**): from the DNA samples to the final data, each step, including sample test, PCR, library preparation, and sequencing, influences the quality of the data, and data quality directly impacts the analysis results. To guarantee the reliability of the data, quality control (QC) is performed at each step of the procedure. The workflow is as following:

Figure 21. Workflow for lung microbiome sequencing



2.6.1 PCR amplification and purification

For the 16S rRNA gene sequencing, V3-V4 region was amplified by specific primer set (V3F: 5'-CCTACGGGNGGCWGCAG-3', V4R: 5'- GACTACHVGGGTAT CTAATCC -3') according to the 16S Metagenomic Sequencing Library Preparation procedure (Illumina). In brief, 12.5 ng of gDNA was used for the PCR reaction carried out with KAPA HiFi HotStart ReadyMix (Roche) under the PCR condition: 95°C for 3 minutes; 25 cycles of: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds;

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72°C for 5 minutes and hold at 4°C. The PCR products were monitored on 1.5% agarose gel. Samples with bright main strip around 500bp were chosen and purified by using the AMPure XP beads for the following library preparation [103].

2.6.2 Library preparation and sequencing

The Sequencing library was prepared according to the 16S Metagenomic Sequencing Library Preparation procedure (Illumina). In brief, a secondary PCR was performed by using the 16S rRNA V3-V4 region PCR amplicon and Nextera XT Index Kit with dual indices and Illumina sequencing adapters (Illumina). The indexed PCR product quality was assessed on the Qubit 4.0 Fluorometer (Thermo Scientific) and Qsep100TM system. Equal amount of the indexed PCR product was mixed to generate the sequencing library. At last, library was sequenced on an Illumina MiSeq platform and paired 300-bp reads were generated [104].

2.7 Lung microbiome analysis

The raw paired-end 16S rRNA sequencing data files were initially analyzed using QIIME 2 with the DADA 2 plugin (version 2022.2) [105] to generate nonchimeric Amplicon sequence variants (ASVs). Taxonomic assignment was performed using the naive Bayesian classifier built-in R package DADA2 (assign Taxonomy function; version 1.22.0) [52] and the curated SILVA 138.1 database (https://github.com/mammerlin/U16SDD2B/tree/main/Curated%20DB/Curated%20SIL

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VA). Furthermore, the taxonomy of ASVs assigned as NA (Not Available) at the species level in the DADA2 assignment results was determined using DD2B (https://github.com/mammerlin/U16S-DD2B/tree/main/DD2B) with BLAST+ (MGEGABLAST; version 2.12.0) [106].

Finally, the raw ASV abundance was aggregated into the corresponding taxon after taxonomic assignment. The aggregated taxon abundances were then rarified to the minimum number of reads present in the samples for subsequent analyses.–For the subsequent data analysis, we use the R software (version 4.1.2) and the Phyloseq [107], vegan [108], microViz [109], and ggplot2 [110] packages. Alpha diversity measurements were calculated using the Shannon index. Beta diversity analysis was performed through a PCoA of Bray–Curtis matrices. Nonparametric statistical analyses, including Wilcoxon rank-sum tests and Kruskal–Wallis tests, were used to compare the relative abundance of taxa and alpha diversity of the groups. Adonis permutational analysis of variance tests were performed to compare the beta diversity between the groups. Pairwise differences in beta diversity were also analyzed by conducting a permutational analysis of multivariate dispersions (Betadisper function in vegan, 999 permutations). Spearman's correlation test was used to analyze the correlations between clinical variables and selected taxa. Statistical significance was determined using a two-sided *P* value of <0.05 for diversity analysis or a Benjamini–Hochberg adjusted *P* value of <0.05 for multiple

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testing analysis. DESeq2 (version 1.34.0) [111] with “poscounts” size factor estimation and default settings was used to identify differentially abundant taxa between groups of samples. Stacked bar plots of the most abundant taxa were plotted with microViz and ggplot2 packages.

2.8 Statistical analysis

In this study, continuous variables were presented as mean ± standard deviation (SD) for parametric data and as medians with inter-quartile ranges (IQR) for nonparametric data. For comparing groups, we used the independent samples t-test for parametric data and the Mann-Whitney test for nonparametric data. Categorical variables were analyzed using the chi-square test or Fisher’s exact test, depending on the data suitability. These statistical analyses were conducted using SPSS software (version 18.0, IBM). All tests were two-sided, and a P value of less than 0.05 was considered indicative of statistical significance. The methodology for microbiome analysis and other statistical procedures are detailed as previously described.

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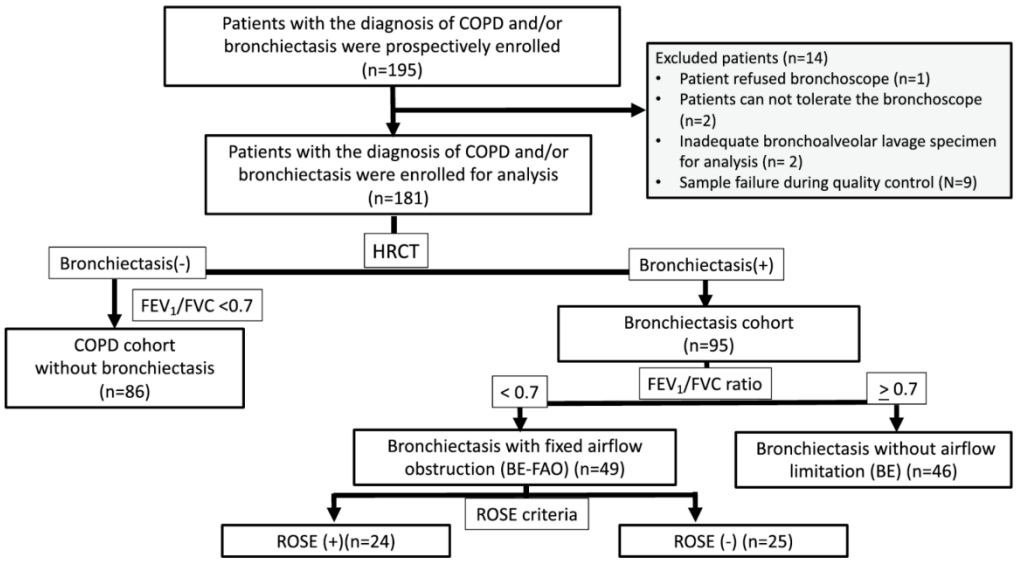
Chapter 3. Results

3.1 Clinical characteristics of patients with bronchiectasis and COPD

Of the 195 consecutively stable patients with COPD and/or bronchiectasis initially enrolled, 181 were included in the final analysis. The study cohort comprised 86 patients with COPD, 46 patients with BE, and 49 patients with BE-FAO (**Figure 22**) [112].

Figure 22. The workflow of patients recruited our the study



Their demographic and clinical characteristics are summarized in **Table 3**. Compared with the COPD group, the BE-FAO group had higher neutrophil counts in the blood and BAL samples, higher C-reactive protein (CRP) levels (**Table 4**), more exacerbation episodes in the past year, and a higher frequency of prior tuberculosis. Additionally, the BE-FAO group had higher bronchiectasis severity, more extensive emphysema, worse airway symptoms, higher CRP levels, lower lung function indices, and greater reliance on bronchodilators than the BE group.

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Table 3. Clinical characteristics of study participates (N = 181)

Clinical factors/variables	COPD	BE	BE-FAO	P value BE-FAO vs COPD	P value BE-FAO vs BE
Number	**86**	**46**	**49**		
Age, years, median (IQR)	67.9(63.1-77.3)	67.1(59.3-75.4)	73.6(62.4-78.9)	0.2	0.044*
Gender, Man, n (%)	83(96.5)	20(43.5)	35(71.4)	<0.001*	0.005*
BMI, median (IQR)	24.2(22.1-26.2)	21.2(18.3-24.2)	22.4(3.8)	0.001*	0.468
Smoking status, n (%)					
Nonsmoker	7(8.1)	33(71.7)	25(51.0)	<0.001*	0.031*
Ex-smoker or current smoker	79(91.9)	13(28.3)	24(49.0)		
Lung function test, median (IQR)					
FEV_1/FVC (%)	63.7(53.1-68.8)	78.0(74.9-80.0)	64.1(58.7-66.9)	0.913	<0.001*
FEV_1 (%)	73.0(59.0-85.0)	91.7(77.7-104.6)	70.0(53.7-79.8)	0.155	<0.001*
FVC (%)	91.8(82.3-107.5)	94.7(79.3-104.5)	86.4(73.9-99.6)	0.055	0.11
Bronchodilator reversibility, n (%)	17(19.8)	4(8.7)	6(12.2)	0.191	0.411
Emphysema score, median (IQR)					
LAV<-930(HU) (%)	8.5(2.8-19.9)	2.67(0.83-8.11)	5.5(2.7-16.4)	0.224	0.003*
Radiological severity of bronchiectasis					
Bronchiectasis involved lobes, median (IQR)	—	3.0(2.0-4.0)	4.0(3.0-5.0)	n.a	0.001*
Modified Reiff score, median (IQR)	—	3.5(2.0-4.0)	5.0(3.0-6.0)	n.a	0.010*
Bronchiectasis severity index (BSI), median (IQR)	—	6.0(3.0-9.0)	8.0(6.0-10.0)	n.a	0.013*
Mild (0-4), n (%)	—	18(39.1)	6(12.2)		
Moderate (5-8), n (%)	—	15(32.6)	20(40.8)	n.a	0.009*
Severe (≥9), n (%)	—	13(28.3)	23(46.9)		
mMRC (dyspnea scale), n (%)					
0-1	50(58.1)	34(73.9)	24(49.0)	0.198	0.011*
≥ 2	36(41.9)	12(26.1)	25(51.0)		
CAT score (symptoms score), n (%)					
<10	66(76.7)	33(71.7)	35(71.4)	0.314	0.577
≥10	20(23.3)	13(28.3)	14(28.6)		
Exacerbation in prior yr					
Low risk: 0-1 time /year	79(91.9)	38(82.6)	38(77.6)	0.020*	0.361
High risk: ≥ 2 times /year	7(8.1)	8(17.4)	11(22.4)		

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Comorbidities, n (%)					
Cardiovascular disease	51(59.3)	15(32.6)	21(42.9)	0.048*	0.207
Diabetes mellitus	20(23.3)	7(15.2)	5(10.2)	0.047*	0.335
Chronic kidney disease	13(15.1)	7(15.2)	8(16.3)	0.518	0.554
Chronic liver disease	17(19.8)	11(23.9)	6(13.2)	0.191	0.112
Gastroesophageal reflux disease	53(61.6)	23(50.0)	23(46.9)	0.07	0.463
Obstructive sleep apnea	8(9.3)	2(4.3)	4(8.2)	0.546	0.369
History of tuberculosis infection	3(3.5)	10(21.7)	14(28.6)	<0.001*	0.229
Autoimmune disease	1(1.2)	6(13.0)	2(4.1)	0.298	0.114
Inhalation therapy, n (%) at baseline					
Short-acting bronchodilator or none	13(15.1)	34(73.9)	10(20.4)		
Monotherapy (LAMA or LABA)	19(22.1)	4(8.7)	4(8.2)		
Dual Therapy (ICS + LABA)	2(2.3)	0(0)	0(0)	0.214	<0.001*
Dual bronchodilators (LAMA+LABA)	38(44.2)	7(15.2)	26(53.1)		
Triple therapy	14(16.3)	1(2.2)	9(18.4)		
Inhaled corticosteroid (ICS)	16(18.6)	1(2.2)	9(18.4)	0.583	0.010*

Data are presented as No. (%) or median (interquartile range), unless otherwise indicated; n.a.: not available

For each row, data are either % with p-values from t test or Fisher's exact tests between the two groups, median (IQR) with p-values from Mann-Whitney tests; *p <0.05. BE = Bronchiectasis without fixed airflow obstruction; BE-FAO=Bronchiectasis with fixed airflow obstruction; BMI=Body Mass Index; COPD=Chronic obstructive pulmonary disease; FEV_1=forced expiratory volume in 1 sec; FVC = forced vital capacity; LAV = low-attenuation volume; HU: Hounsfield unit; CAT = COPD Assessment Test; mMRC = modified Medical Research Council; LAMA=long-acting muscarinic antagonist; LABA=Long-acting β2 Sympathomimetic Agonists; ICS=Inhaled corticosteroid

Table 4. Clinical samples analysis of study patients (N=181)

Laboratory data	COPD	BE	BE-FAO	P value BE-FAO vs COPD	P value BE-FAO vs BE
Number	**86**	**46**	**49**		
Blood sample, median (IQR)					
Hemoglobin (g/dl)	15.1(13.6-15.8)	13.5(12.3-14.2)	13.8(12.8-14.8)	<0.001*	0.142
Platelet count (K/μl)	214.5(183.0-250.2))	228(193-288)	243(208-285)	0.004*	0.441
White blood cell counts (K cells/mm^3)	6.41(4.97-7.49)	6.64(4.91-8.22)	7.51(5.41-9.25)	0.010*	0.058

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Neutrophil(%)	59.2(52.9-64.8)	61.4(53.3-68.5)	64.4(57.2-70.8)	0.001*	0.120
Eosinophil(%)	2.8(1.6-4.3)	2.1(1.2-3.4)	2.6(1.4-4.1)	0.263	0.292
<2%, n(%)	26(30.2)	22(47.8)	19(38.8)	0.205	0.247
≥2%, n(%)	60(69.8)	24(52.2)	30(61.2)		
Eosinophil counts (cells/mm^3)	173.8(101.1-287.4)	134.7(65.4-235.3)	162.1(126.4-271.0)	0.770	0.092
C-reactive protein (mg/dL)	0.15(0.06-0.31)	0.14(0.04-0.68)	0.42(0.23-0.88)	0.001*	0.004*
BAL samples, median (IQR)					
Macrophage %	83.5(76.3-89.3)	87.1(77.2-89.6)	85.9(81.0-91.4)	0.056	0.175
Neutrophils %	1.4(0.6-2.5)	1.2(0.7-2.7)	2.1(0.9-5.2)	0.024*	0.076
Eosinophils %	2.9(1.8-5.4)	2.3(1.1-4.3)	1.8(1.2-2.9)	0.003*	0.290
Lymphocyte %	8.8(6.4-15.1)	7.4(3.1-16.6)	8.0(3.9-10.7)	0.013*	0.655
BAL sample, median (IQR)					
Eotaxin (pg/ml)	1.6(1.0-5.4)	1.3(0.7-3.5)	1.9(0.9-3.5)	0.873	0.375
IL-1β (pg/ml)	4.2(2.1-9.2)	13.1(3.7-100.4)	56.0(4.6-352.6)	<0.001*	0.095
IL-6 (pg/ml)	10.2(2.5-21.0)	16.6(5.4-45.9)	31.1(6.4-69.7)	<0.001*	0.175
IL-18 (pg/ml)	31.1(20.0-52.5)	36.3(25.4-52.5)	38.3(23.8-48.9)	0.385	0.754
IL-8 (pg/ml)	201.6(64.4-377.0)	423.6(131.0-1453.0)	958.5(224.8-2616.5)	<0.001*	0.048*
TNF-α (pg/ml)	4.8(2.5-9.5)	7.9(3.9-27.7)	13.2(5.6-37.7)	<0.001*	0.074
MCP-1(pg/ml)	171.9(86.7-311.4)	228.2(97.7-567.0)	259.5(165.1-553.4)	0.001*	0.461
NETs (pg/ml)	0.36(0.18-0.63)	0.57(0.21-1.04)	1.01(0.55-2.75)	<0.001*	0.005*
Conventional culture of BAL samples					
Klebsiella pneumoniae, n(%)	14(30.0)	13(28.3)	18(36.7)	0.327	0.255
Pseudomonas aeroginosa, n (%)	1(3.2)	12(26.1)	16(32.7)	<0.001*	0.317
Staphylococcus aureus, n(%)	12(26.1)	4(8.7)	11(22.4)	0.436	0.059
Haemophilus influenzae, n(%)	6(13.0)	7(15.2)	3(6.1)	0.577	0.134
Non-tuberculosis mycobacterium, n(%)	3(6.5)	7(15.2)	7(14.3)	0.091	0.563
Other bacterial pathogens, n(%)	20(43.5)	21(45.7)	20(40.8)	0.408	0.394
Potential pathogenic bacteria colonization, n(%)	35(76.1)	38(82.6)	44(89.9)	0.008*	0.263
Aspergillus species, n(%)	3(6.5)	3(6.5)	5 (10.2)	0.543	0.393
Candida species, n(%)	5(10.9)	8(17.4)	12(24.5)	0.132	0.276

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For each row, data are either % with p-values from t test or Fisher's exact tests between the two groups, median (interquartile range, IQR) with p-values from Mann-Whitney tests; *p <0.05. BAL = Bronchoalveolar lavage; BE = Bronchiectasis without fixed airflow obstruction; BE-FAO = bronchiectasis with fixed airflow obstruction; COPD = Chronic obstructive pulmonary disease;IL-1β=interleukin [IL]-1β; IL-6=interleukin [IL]-6; IL-8= interleukin [IL]-8; IL-18=interleukin [IL]-18; MCP-1=Monocyte chemoattractant protein-1; NETs= Neutrophil extracellular traps; TNF-α=tumor necrosis factor [TNF]-α.

3.2 Negative controls and decomtam method results

Before lung microbiome analysis, we addressed potential background contaminations, we performed DNA extraction and PCR amplification for the biological control (oral washing fluid) and background negative controls (including bronchoscope channel washing fluid, sterile saline and reagents) obtained from study participants from the COPD, bronchiectasis without airflow obstruction (BE), and bronchiectasis with fixed airflow obstruction (BE-FAO) groups in parallel to account for potential contamination. In brief, a total of 78 oral washing control (OWC) samples, 5 bronchoscope channel washing (BCW) fluid samples, 5 sterile normal saline control (NSC), phosphate buffered (PBS) control, 5 extraction kit control (EKC) and 5 non-template control (NTC) were processed for 16S rRNA sequencing.

During the microbiome analysis of 181 BAL samples collected from stable patients, a total of 7771 amplicon sequence variants (ASV) were consolidated to 1750 taxa. To remove the potential contaminations, the combined method in R package Decontam (v1.16.0) [113] with background negative controls were performed and 65 and 20

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potential background contaminate species were identified and removed from BAL and OWC samples, resulting in 1685 and 800 species, respectively. Afterward, we performed a rarefaction analysis of 181 BAL samples to obtain the same library size (read count =20346). To filter out rare taxa, the remaining 1624 taxa found in fewer than 10% of BAL samples were removed and among ASV annotated to specie, we detected 295 taxa for final analysis in BAL samples (**Table 5**).

Table 5. Decomtam flow and sequencing data[a,]

Samples	No. ASVs	No. Species	No. Species of Contaminant	No. Species after decontam	No. Species after rarefaction	No. Species after filter out taxon with prevalence < 10%
181 BAL	7771	1750	65	1685	1624	295
78 OWC	3951	820	20	800	-	-
28 NC	1378	792	-	-	-	-

a. Raw sequencing data upload to NCBI SRA --**Project ID: PRJNA924101**

https://dataview.ncbi.nlm.nih.gov/object/PRJNA924101?reviewer=6aivli35eho5jdrfoatvvpbf10

ASV= Amplicon sequence variants (ASVs); BAL= Bronchoalveolar lavage; NC=Negative control; OWC=oral washing control.

Before removing the contaminants, the BAL and NC showed similar alpha-diversity (**Figure 23A**), which were significantly higher than OWC samples (P<0.05). The significant differences in the beta diversity (**Figure 23B**) of microbiome communities among the BAL, OWC and NC samples (R^2 =0.331, P=0.01, ADONIS permutational multivariate analysis of variance (PERMANOVA)) were noted.

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Figure 23 The alpha diversity (A) and beta diversity (B) of bronchoalveolar lavage (BAL), oral washing control (OWC) and negative control (NC) samples before decontam method.

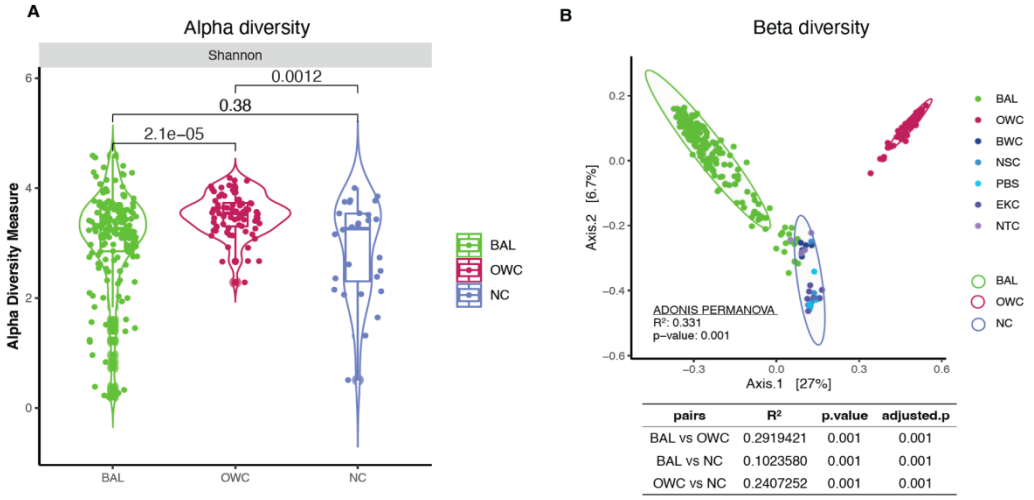

Figure 23. The alpha diversity (A) and beta diversity (B) of bronchoalveolar lavage (BAL), oral washing control (OWC) and negative control (NC) samples before decontam method. BAL samples (N=181, green dots), OWC samples (N=78, red dots) and NC samples including Bronchial washing control (BWC) (n=5, deep blue dots), Normal saline control (NSC) (n=5, light blue dots), Phosphate buffered saline (PBS) control (n=5, cyan blue dots), Extraction kit control (EKC) (n=8, deep purple dots), Non-Template control (NTC) (n=5, light purple dots).

After the decontam method was performed, microbiome analysis revealed significant differences in alpha diversity between the BAL and OWC samples ($P < 0.001$, **Figure 24A**).; and a principal coordinates analysis (PCoA) revealed a significant separation of microbial communities between the BAL and OWC samples (ADONIS PERMANOVA $R^2 = 0.293$, $P = 0.001$, **Figure 24B**), indicating that the microbiome compositions of the BAL and OWC samples were significantly different. However, we still could not exclude the possibility that some lung microbiota of the BAL samples overlapped with pharyngeal taxa because of subclinical microaspiration or the procedural effect [13, 58]. However, no established standards exist for sampling lung microbiome

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without carry over of upper airway microbes [35,57], and BAL sampling does present a theoretical risk of exposure to pharyngeal microbiota [35]. Therefore, our procedure protocol for the negative control samples was implemented to minimize background contamination.

Figure 24 The alpha diversity and beta diversity of bronchoalveolar lavage (BAL) (N=181) and oral washing control (OWC) (N=78) samples after removing the background contamination taxa

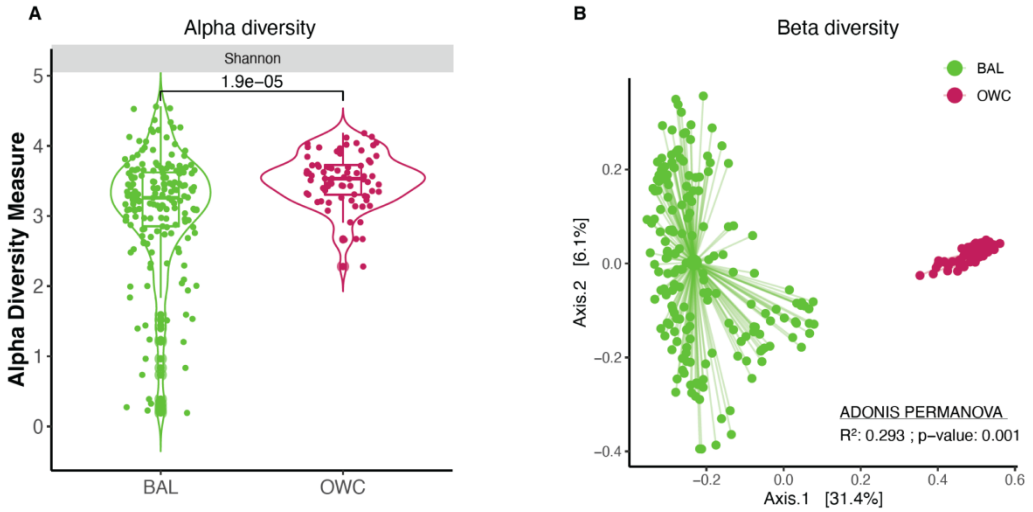

Figure 24 The alpha diversity and beta diversity of bronchoalveolar lavage (BAL) (N=181) and oral washing control (OWC) (N=78) samples after removing the background contamination taxa. The microbiome analysis showed that BAL samples and OWC displayed significantly different. A, alpha-diversity (P<0.001). B, Principal coordinates analysis (PCoA) showed significant separation microbial communities between the BAL and OWC samples (R2=0.293, P-value =0.001).

3.3 Lung microbiome comparison between BE-FAO, BE, and COPD groups

In our study, alpha diversity in the BE-FAO and BE groups was significantly lower than that in the COPD group ($P < 0.05$, **Figure 25A**). Beta diversity significantly differed between the BE-FAO, BE, and COPD groups ($R^2 = 0.025$, $P = 0.001$, ADONIS PERMANOVA). Notably, the COPD group differed considerably from the BE-FAO (R^2

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= 0.0203, adjusted P = 0.0015) and BE groups (R^2 = 0.0219, adjusted P = 0.0015). However, the diversity indices were similar in the BE and BE-FAO groups, suggesting substantial overlaps in their microbiome profiles (R^2 = 0.0107, adjusted P = 0.4370, detailed in **Figure 25B**).

Figure 25 Alpha diversity (A) and beta diversity (B) of BAL microbiome profiles in COPD, BE and BE with FAO

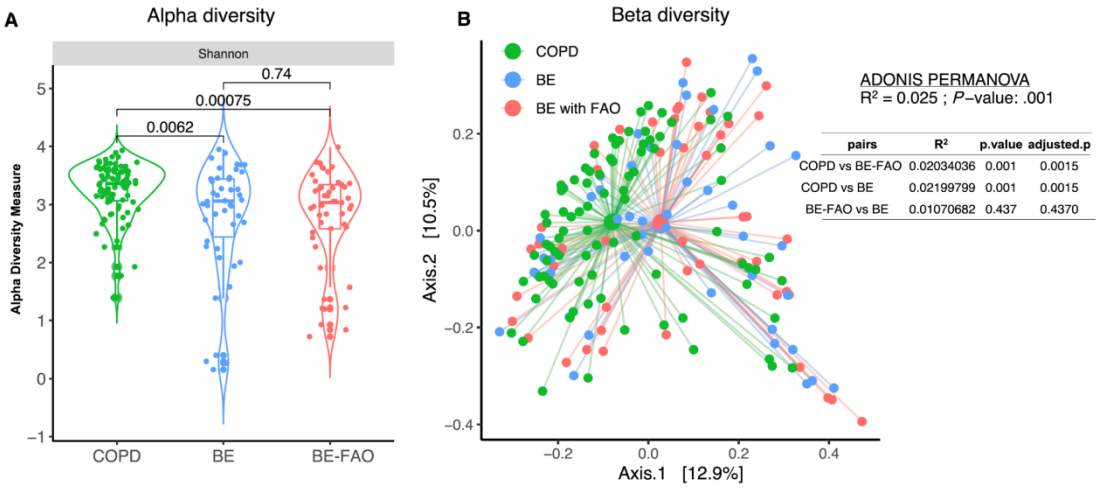

Figure 25. Alpha diversity (A) and beta diversity (B) of BAL microbiome profiles in COPD, BE and BE with FAO. (A). Patients in BE and BE-FAO groups displayed similar Shannon diversity, which were significantly lower than those with COPD alone. (B). The pairwise values using Bray-Curtis distance and principal coordinates analysis (PCoA) to measure the beta diversity between COPD, BE-FAO and BE groups. BE=Bronchiectasis without fixed airflow obstruction; BE-FAO= Bronchiectasis with fixed airflow obstruction; COPD=Chronic obstructive pulmonary disease.

At the phylum level, patients with BE-FAO had higher *Proteobacteria* and lower *Firmicutes* levels than patients with COPD (**Figure 26**). No significant differences were found in four major phyla between the BE and BE-FAO groups. A detailed analysis of the ASVs annotated to species revealed that six taxa were significantly enriched in the

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COPD group, in contrast to the higher levels of *Pseudomonas aeruginosa* in the BE-FAO group (**Figure 27**).

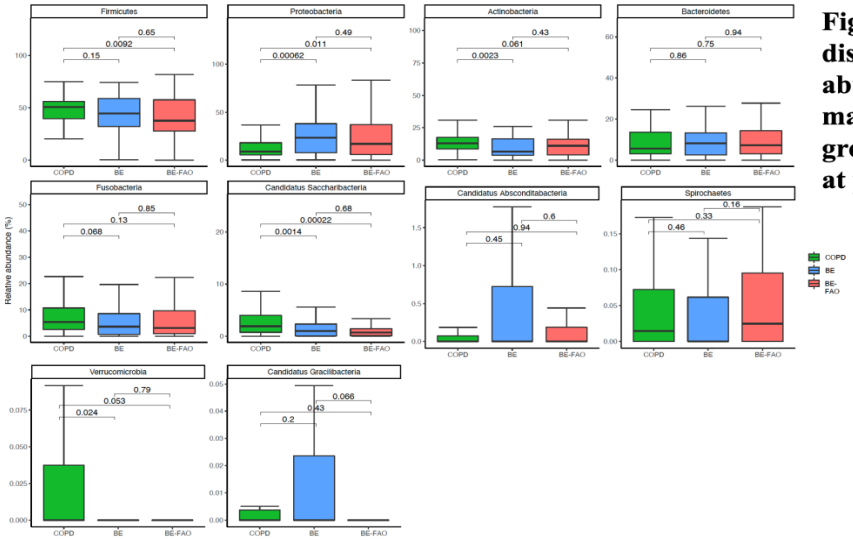

Figure 26. The distribution of relative abundance of top 10 major taxonomic groups in three groups at phylum level.

Figure 26. The distribution of relative abundance of top 10 major taxonomic groups in three groups at phylum level. The patients with BE-FAO had a higher relative abundance of *Proteobacteria*(p=0.011) and lower abundance of *Firmicutes*(p=0.0092) relative to the patients with COPD. No significant difference was observed in the proportions of the four major phyla in BE and BE-FAO. BE=Bronchiectasis without fixed airflow obstruction; BE-FAO= Bronchiectasis with fixed airflow obstruction; COPD=Chronic obstructive pulmonary disease.

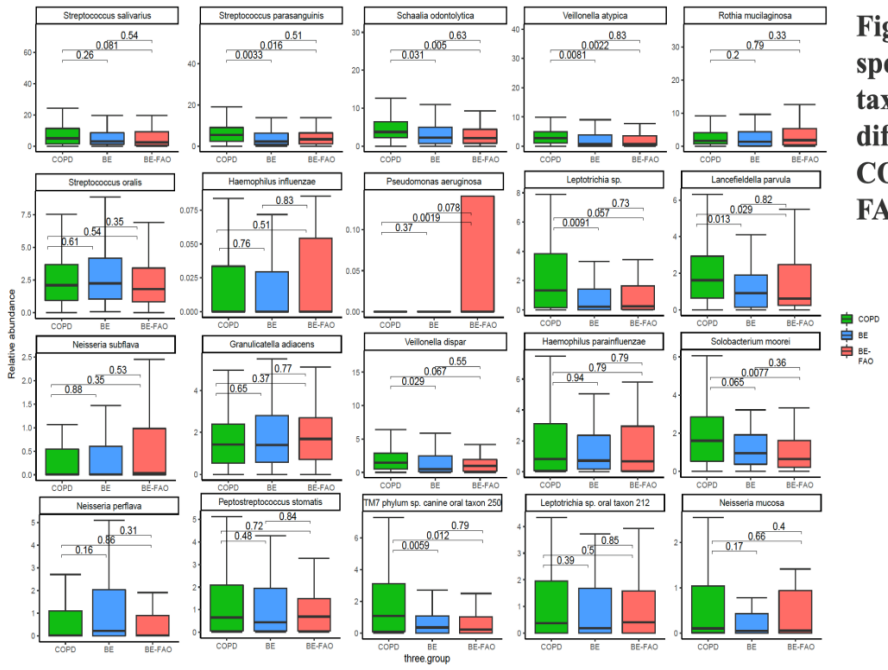

Figure 27. Highlights species-level taxonomic distribution differences between COPD, BE, and BE-FAO patients.

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Figure 27. Highlights species-level taxonomic distribution differences between COPD, BE, and BE-FAO patients. Among ASV annotated to specie, the COPD group showed higher prevalence of Streptococcus parasanguinis, Schaalia odontolytica, Veillonella atypica, Lancefieldella parvula, Solobacterium moorei, and TM7 phylum sp canine oral taxon 250, while Pseudomonas aeruginosa was more abundant in the BE-FAO group. Wilcoxon rank-sum test was used to compare the relative abundance of taxa. BE=Bronchiectasis without fixed airflow obstruction; BE-FAO= Bronchiectasis with fixed airflow obstruction; COPD=Chronic obstructive pulmonary disease.

These microbial distributions were consistent with the conventional culture results detailed in **Table 4**. The concordance rate between the results of 16S rRNA gene sequencing and culture-based identification was 64.9% at the species level and 66.7% at the genus level (**Table 6**).

Table 6 The comparison of conventional culture and 16S rRNA gene sequencing results

Taxa name	Conventional Culture	16S rRNA sequencing		
	n	n	Mean relative abundance (%)	16S sequencing detection rate (%) (compared to culture)
Species				
Klebsiella pneumoniae	57	39	6.912	68.4
Staphylococcus aureus	32	20	1.899	62.5
Pseudomonas aeruginosa	30	23	17.859	76.7
Haemophilus influenzae	16	15	32.241	93.8
Haemophilus parahaemolyticus	10	8	1.299	80.0
Haemophilus parainfluenzae	10	10	2.448	100.0
Escherichia coli	7	7	24.238	100.0
Mycobacterium chimaera intracellulare group	7	0	NA	0.0
Enterobacter cloacae complex	4	0	NA	0.0
Klebsiella oxytoca	4	0	NA	0.0
Stenotrophomonas maltophilia	4	1	0.244	25.0
Streptococcus agalactiae	4	3	0.023	75.0
Streptococcus pneumoniae	4	2	3.547	50.0

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Klebsiella variicola	3	1	0.324	33.3
Acinetobacter pittii	2	0	NA	0.0
Haemophilus haemolyticus	2	1	0.027	50.0
Serratia marcescens	2	0	NA	0.0
Streptococcus anginosus	2	2	1.004	100.0
Acinetobacter baumannii	1	0	NA	0.0
Acinetobacter junii	1	0	NA	0.0
Alcaligenes faecalis	1	0	NA	0.0
Beta-streptococcus	1	0	NA	0.0
Bordetella sp.	1	0	NA	0.0
Chryseobacterium gleum	1	0	NA	0.0
Comamonas terrigena	1	0	NA	0.0
Haemophilus paraphrohaemolyticus	1	0	NA	0.0
Klebsiella aerogenes	1	0	NA	0.0
Moraxella_sg_Branhamella catarrhalis	1	1	38.161	100.0
Mycobacterium colombiense	1	0	NA	0.0
Mycobacterium interjectum	1	0	NA	0.0
Neisseria gonorrhoeae	1	1	17.283	100.0
Pasteurella multocida	1	1	28.508	100.0
Proteus vulgaris	1	0	NA	0.0
Raoultella ornithinolytica	1	0	NA	0.0
Streptococcus constellatus	1	1	0.618	100.0
Streptococcus dysgalactiae	1	1	0.203	100.0
Wautersiella falsenii	1	0	NA	0.0
	211	**137**		**64.9%**
Genus				
Mycobacterium (Mycobacterium chimaera intracellulare group, Mycobacterium species(NTM),Mycobacterium avium intracellulare complex,Mycobacterium species, Mycobacterium tuberculosis complex)	6	2	5.905	33.3
Nocardia species	3	2	4.938	66.7

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Acinetobacter species	2	2	1.584	100.0
Achromobacter species	1	1	1.635	100.0
Aeromonas species	1	0	0.000	0.0
Cunninghamella species	1	0	0.000	0.0
Pseudomonas species	1	1	0.029	100.0
Rhizopus species	1	0	0.000	0.0
	21	**14**		**66.7%**

Additionally, the detailed stacked plot in **Figure 28** illustrates the relative abundance of the aforementioned taxa in the COPD, BE, and BE-FAO groups.

Figure 28. Stacked plot of relative abundance of taxa at the species level in each sample in three groups

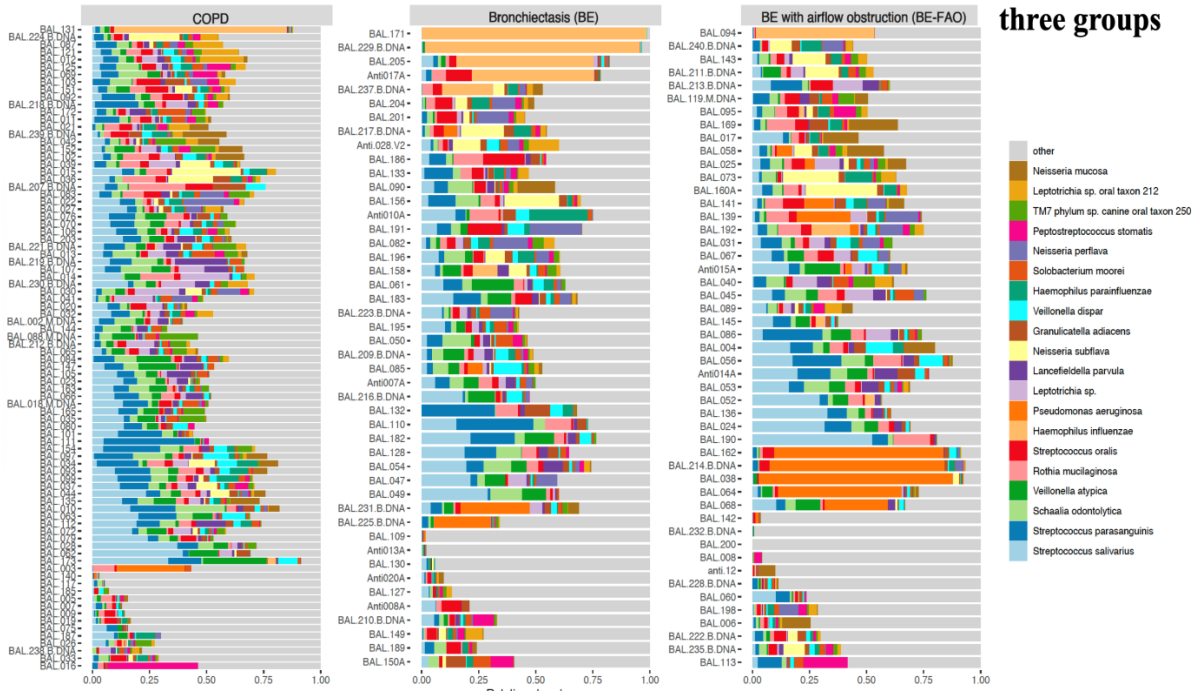

Figure 28. Stacked plot of relative abundance of taxa at the species level in three groups. COPD (n=86), BE (n=46) and BE-FAO (n=49) groups. BE=Bronchiectasis without fixed airflow obstruction; BE-FAO= Bronchiectasis with fixed airflow obstruction; COPD=Chronic obstructive pulmonary disease.

After adjustment for gender and smoking status, our differential abundance analysis using DESeq2 indicated that the groups differed in their microbiome profiles (adjusted $P < 0.05$ and fold change >2). At the species level, the BE group had enriched *Pseudomonas aeruginosa* and *Haemophilus influenzae*, in contrast to the high levels of commensal

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species in the COPD group (**Figure 29A**). Moreover, unlike the COPD group, the BE-FAO group had a predominance of *Pseudomonas aeruginosa, Limosilactobacillus fermentum, Ligilactobacillus salivarius*, and *H. influenzae* (**Figure 29B)**.

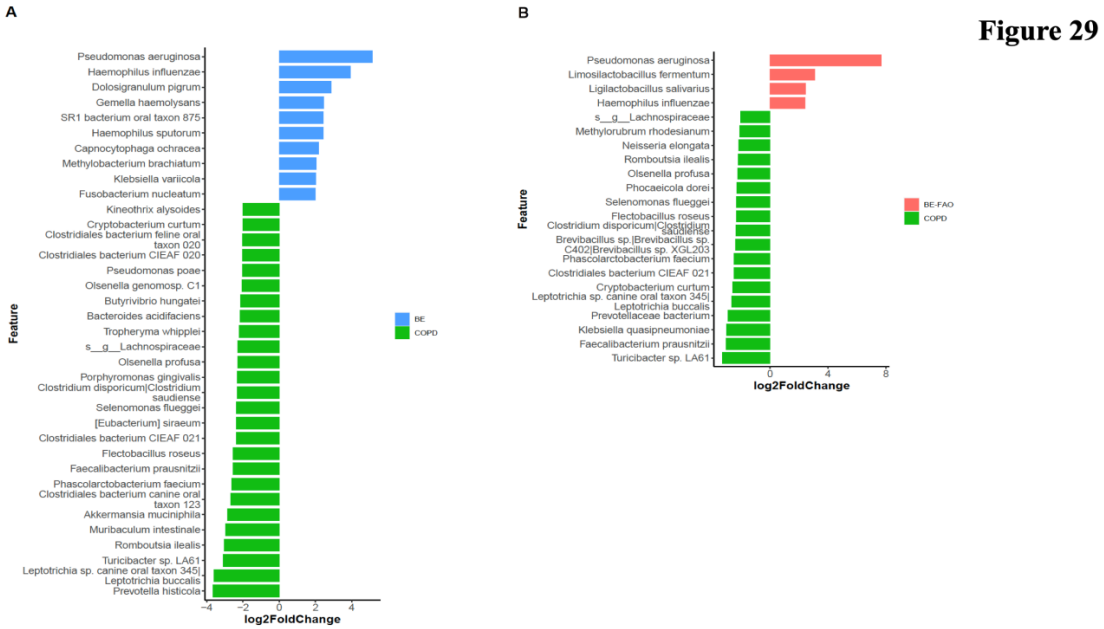

Figure 29

Figure 29. The differential abundance of lung microbiome analysis using DEseq2 in COPD, BE and BE-FAO groups (adjust gender and smoking status). The different taxonomic levels (adjusted $P < 0.05$ and fold change > 2.0) at species level in BE versus COPD groups (**A**) and in BE-FAO versus COPD groups (**B**).BE=Bronchiectasis without fixed airflow obstruction, BE-FAO=Bronchiectasis with fixed airflow obstruction, COPD=Chronic obstructive pulmonary disease

3.4 Bronchiectasis with FAO exhibits neutrophilic inflammation and specific microbiota compared to BE and COPD

In comparison with patients having COPD alone, those with BE-FAO exhibited significantly elevated levels of BAL neutrophils and increased concentrations of neutrophilic inflammatory cytokines: Interleukin (IL)-1β, IL-6, IL-8, Monocyte Chemoattractant Protein-1 (MCP-1), and Tumor Necrosis Factor-alpha (TNF-α), as

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detailed in **Table 4.** Additionally, the BE-FAO group displayed higher levels of IL-8 and NETs compared to the BE group, despite presenting similar lung microbiome profiles. Further analysis, illustrated in **Figure 30**, explores the correlations between clinical variables and specific lung bacterial taxa across the COPD, BE, and BE-FAO groups. Notably, *Pseudomonas aeruginosa* was positively correlated with airway neutrophilic cytokines and was associated with increased bronchiectasis severity (BSI score) and lower BMI in the BE-FAO group.

Figure 30. Heatmap showing spearman correlation between clinical variables and microbiome in COPD, BE and BE-FAO groups

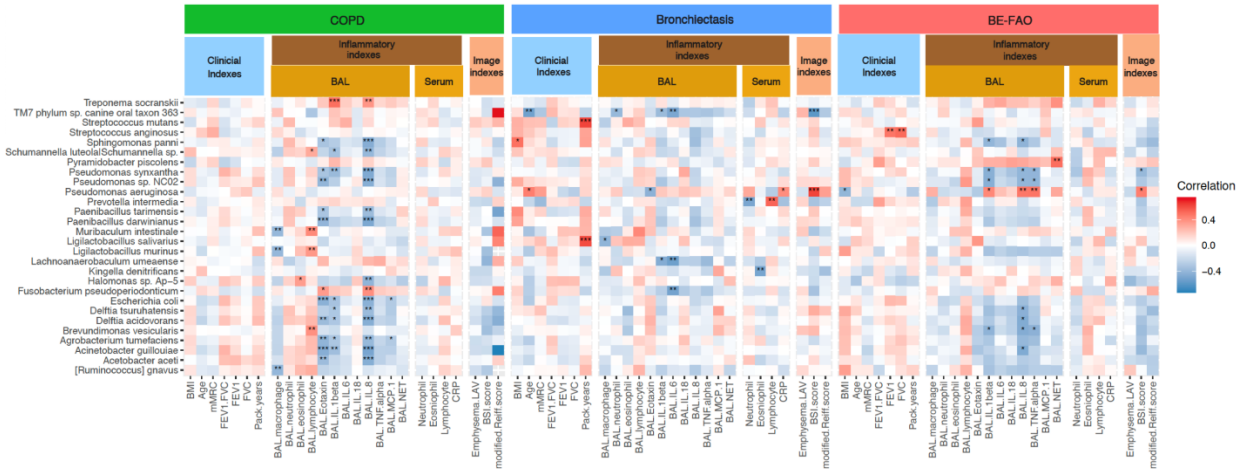

Figure 30 Heatmap showing spearman correlation between clinical variables and microbiome in COPD, BE and BE-FAO groups. Clinical variables are grouped into three categories: clinical indexes, inflammatory indexes, and imaging indexes. Only those taxa that displayed at least one significant correlation ($q < .01$, following FDR correction) were selected. The color-coded matrix represents the Spearman correlation coefficient, with red indicating a positive correlation and blue indicating a negative correlation. FDRs are denoted: *$q < 0.05$; **$q < 0.01$; ***$q < 0.001$. BAL= Bronchoalveolar lavage; BE=Bronchiectasis without fixed airflow obstruction; BE-FAO= Bronchiectasis with fixed airflow obstruction; BMI=Body Mass Index; BSI=Bronchiectasis severity index; CAT=COPD Assessment Test; COPD= Chronic obstructive pulmonary disease; CRP=C-reactive protein; FDR=False discovery rate; FEV_1=forced expiratory volume in 1 sec; FVC=forced vital capacity; LAV=low-attenuation volume; mMRC=modified Medical Research Council; IL-1β=interleukin [IL]-1β; IL-6=interleukin [IL]-6; IL-8=

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interleukin [IL]-8; IL-18=interleukin [IL]-18; MCP-1=Monocyte chemoattractant protein-1; NETs= Neutrophil extracellular traps; TNF-α=tumor necrosis factor [TNF]-α.

3.5 Differences in clinical features, airway inflammation, and lung microbiome among patients with bronchiectasis with FAO according to ROSE criteria

We analyzed clinical variables and clinical outcomes in 49 bronchiectasis patients with FAO, distinguishing between those who met (n=24) and did not meet (n=25) the ROSE criteria, as detailed in **Figure 22** and **Tables 7** and **Table 8.** Patients meeting the ROSE criteria, also known as the "COPD-bronchiectasis association," were predominantly male, often smokers, and generally older. They exhibited a tendency towards COPD-related etiologies, presented with higher dyspnea and emphysema scores on HRCT scans, and showed elevated blood eosinophil and lymphocyte levels (**Figure 31**).

Table 7 Clinical variables and outcomes of patients with bronchiectasis with FAO

	BE with FAO (n=49)		
Clinical factors/variables	**ROSE (+)**	**ROSE (-)**	P value
Number	**24**	**25**	
Age, years, median (IQR)	76.7(69.7-79.8)	70.1(58.0-76.4)	0.026*
Gender, Man, n(%)	24(100)	11(44.0)	<0.001*
BMI (kg/m2), median (IQR)	22.4(20.1-25.4)	20.6(19.5-24.2)	0.150
Smoking status, n (%)			
Nonsmoker	0(0)	25(100)	<0.001*
Current smoker or ex-smoker	24(100)	0(0)	
Smoking pack-years, median (IQR)	41.5(10.0-75.0)	0(0)	<0.001*
Etiologies of Bronchiectasis			
Idiopathic	0 (0)	12(48.0)	<0.001*

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Post-infection			
Pneumonia	5(20.8)	3(12.0)	
NTM or TB	6 (25.0)	10 (40.0)	
COPD	13(54.2)	0(0)	
History of tuberculosis infection, n(%)	6(25.0)	8(32.0)	0.411
Lung function test, median (IQR)			
FEV1/FVC (%)	63.0(49.5-66.5)	64.6(61.2-68.8)	0.147
FEV1 (%)	70.0(42.2-80.9)	69.2(58.4-79.8)	0.555
FVC (%)	85.6(74.7-105.7)	88.6(73.7-97.9)	0.841
Bronchodilator reversibility, n (%)	5(20.8)	1(4.0)	0.086
Radiological severity of Bronchiectasis			
Bronchiectasis involved lobes, median (IQR)	4.0(3.0-5.0)	5.0(3.0-5.5)	0.610
Modified Reiff score, median (IQR)	4.0(3.0-6.0)	5.0(3.0-7.0)	0.419
Bronchiectasis severity index (BSI), median (range)	9.0(7.0-10.0)	8.0(5.5-9.5)	0.231
Mild (0-4), n(%)	2(8.3)	4(16.0)	
Moderate (5-8), n(%)	9(37.5)	11(44.0)	0.538
Severe (≥9), n(%)	13(54.2)	10(40.0)	
Emphysema LAV (%), median (IQR)	11.6(5.6-22.9)	3.2(2.0-5.1)	<0.001*
mMRC (dyspnea scale), n (%)			
0-1	8(33.3)	16(64.0)	0.031*
≥ 2	16(66.7)	9(36.0)	
CAT score (symptoms score), n(%)			
<10	16(66.7)	19(76.0)	0.342
≥10	8(33.3)	6(24.0)	
Exacerbation in the prior year, n(%)			
High risk, ≥2 times/year	18(75.0)	20(75)	0.469
Low risk, 0-1 time/year	6(25.0)	5(20.0)	
Inhalation therapy, n (%) at baseline			
Short-acting bronchodilator or none	4(16.7)	6(24.0)	0.886

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Monotherapy (LAMA or LABA)	2(8.3)	2(8.0)	
Dual bronchodilators (LAMA+LABA)	14(58.3)	12(48.0)	
Triple therapy	4(16.7)	5(20.0)	
Inhaled corticosteroid (ICS)	4(16.7)	5(20.0)	0.527
Clinical outcomes			
Moderate or severe exacerbation	10(41.0)	12(48.0)	0.437
Severe exacerbation	9(37.5)	5(20.0)	0.149

Data are presented as No. (%) or median (interquartile range), unless otherwise indicated; n.a.: nc

For each row, data are either % with p-values from t test or Fisher's exact tests between the two groups, median (interquartile range) with p-values from Mann-Whitney tests; *p <0.05. BE = Bronchiectasis without fixed airflow obstruction; BE-FAO = bronchiectasis with fixed airflow obstruction; BMI = Body Mass Index; COPD = Chronic obstructive pulmonary disease; ROSE=Radiology, Obstruction, Symptoms, Exposure;FEV1 = forced expiratory volume in 1 sec; FVC = forced vital capacity; LAV = low-attenuation volume; CAT = COPD Assessment Test; mMRC = modified Medical Research Council; LAMA = long-acting muscarinic antagonist; LABA = Long-acting β2 Sympathomimetic Agonists; NTM=Non-tuberculosis mycobacteria; ICS = Inhaled corticosteroid; TB=Tuberculosis.

Figure 31

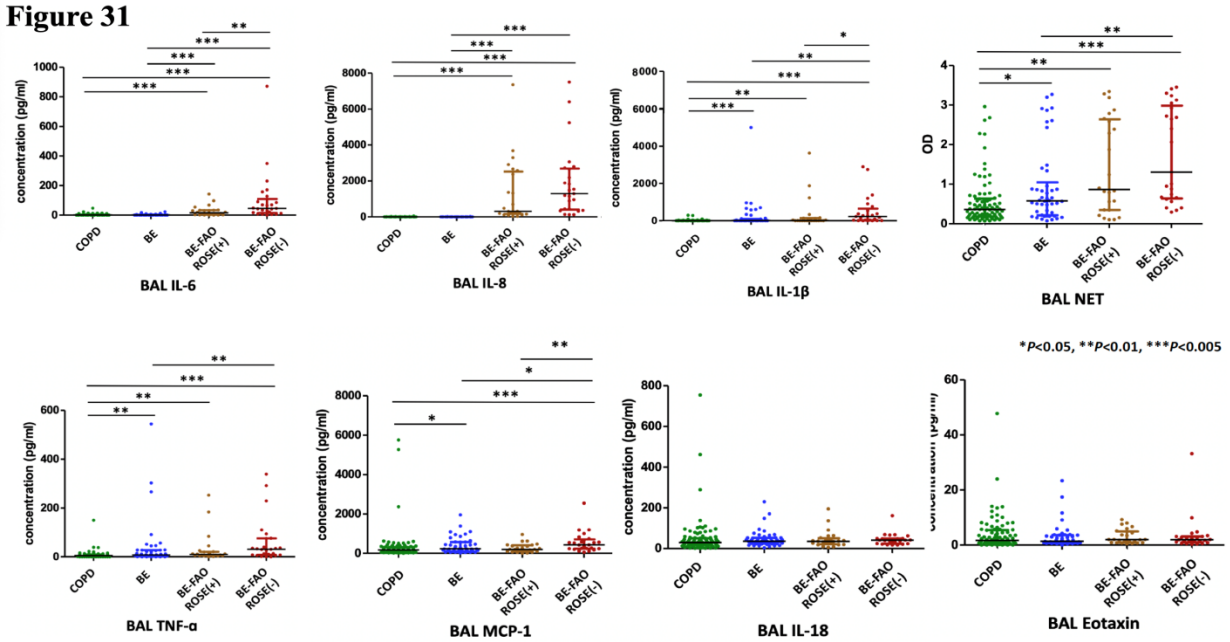

Figure 31. Differences in airway inflammatory profiles based on BAL samples in patients with COPD, BE, BE-FAO ROSE (+), and BE-FAO ROSE (-). The bronchoalveolar lavage (BAL) samples from study subjects were applied for multiplex Immunoassays. (*P<0.05, **P<0.01, ***P<0.005). BAL=bronchoalveolar lavage; BE=Bronchiectasis without fixed airflow obstruction; BE-FAO= Bronchiectasis with fixed airflow obstruction; COPD=Chronic obstructive pulmonary disease; IL-1=Interleukin [IL]-1β, IL-6=Interleukin [IL]-6, IL-18=Interleukin [IL]-18, IL-8=Interleukin [IL]-8; MCP-

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1=Monocyte chemoattractant protein-1; NETs=neutrophil extracellular traps; ROSE=Radiology, Obstruction, Symptoms, Exposure; TNF-α=tumor necrosis factor [TNF]-α.

By contrast, those not meeting the ROSE criteria, who formed a BE-FAO ROSE (−) group, were predominantly female and more likely to have idiopathic etiologies. These patients had significantly elevated levels of neutrophilic inflammatory cytokines, specifically IL-1β, IL-6, and MCP-1, in the BAL samples. Despite these differences, no significant variation was found in lung function indices, bronchiectasis severity, usage of inhaled medications, bacterial culture results, and exacerbation rates between the groups (**Tables 7 and 8**).

Table 8. Laboratory data and BAL culture of patients with bronchiectasis with FAO

	BE with FAO (n=49)		
Laboratory data	**ROSE (+) (n=24)**	**ROSE (-) (n=25)**	P value
Blood sample, median (IQR)			
Hemoglobin (g/dl)	14.2(13.0-14.8)	13.6(12.4-14.8)	0.432
Platelet count (K/μl)	222.0(175.7-270.2)	250.0(219.0-290.5)	0.119
White blood cell counts (K cells/mm^3)	7.55(5.43-9.14)	7.51(5.41-9.35)	0.810
Neutrophil (%)	65.9(56.5-69.7)	63.2(57.9-73.1)	0.849
Eosinophil (%)	3.2(1.8-4.6)	1.9(0.9-2.7)	0.017*
<2 %, n (%)	6(25.0)	13(52.0)	0.049*
≥2%, n (%)	18(75.0)	12(40.0)	
Eosinophil counts	225.7(137.1-303.7)	149.4(81.6-206.62)	0.050
Lymphocyte (%)	22.5(18.1-31.0)	28.1(19.0-33.8)	0.317
Monocyte (%)	6.4(5.9-7.4)	6.2(4.9-7.2)	0.267
C-reactive protein (mg/dL)	0.37(0.15-0.89)	0.45(0.27-0.88)	0.529
BAL samples, median (IQR)			
Macrophage %	86.8(79.2-90.8)	85.1(82.0-93.1)	0.522
Neutrophils %	2.1(0.9-3.5)	2.7(0.8-8.4)	0.390
Eosinophils %	1.8(1.1-2.6)	1.8(1.3-3.0)	0.459
Lymphocyte %	9.3(5.4-14.7)	4.6(2.7-9.2)	0.016*

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Broncholavage sample, median (IQR)			
Eotaxin (pg/ml)	1.9(0.9-4.9)	1.9(0.9-3.1)	0.653
IL-1β (pg/ml)	8.2(3.4-141.2)	230.9(21.7-651.5)	0.016*
IL-6 (pg/ml)	15.6(2.9-33.4)	46.5(16.4-109.4)	0.004*
IL-18 (pg/ml)	35.1(21.22-50.07)	41.1(23.8-48.9)	0.298
IL-8 (pg/ml)	309.8(164.7-2525.2)	1288.3(404.0-2689.4)	0.072
TNF-α (pg/ml)	9.8(5.0-20.9)	31.2(8.0-75.9)	0.050
MCP-1(pg/ml)	199.2(103.5-411.2)	428.3(237.3-712.5)	0.007*
NETs (pg/ml)	0.8(0.3-2.6)	1.3(0.6-2.9)	0.201
Conventional culture of BAL samples			
Klebsiella pneumoniae, n (%)	12(50.0)	6(24.0)	0.055
Pseudomonas aeroginosa, n (%)	6(25.0)	10(40.0)	0.208
Staphylococcus aureus, n (%)	4(16.7)	7(28.0)	0.273
Haemophilus influenzae, n (%)	1(4.2)	2(8.0)	0.516
Non-tuberculosis mycobacterium, n (%)	1(4.2)	6(24.0)	0.055
Asepergillus spp, n (%)	0(0)	5(20.0)	0.028*
Candida spp,	7(29.2)	5(20.0)	0.340
Potential pathogenic bacteria colonization, n (%)	21(87.5)	23(92.0)	0.480

Data are presented as No. (%) or median (interquartile range), unless otherwise indicated

For each row, data are either % with p-values from t test or Fisher's exact tests between the two groups, median (IQR) with p-values from Mann-Whitney tests; *p <0.05. BAL=Bronchoalveolar lavage; BE=Bronchiectasis without fixed airflow obstruction; BE-FAO=Bronchiectasis with fixed airflow obstruction; ROSE=Radiology, Obstruction, Symptoms, Exposure; CRP=C-reactive protein; IL-1β=interleukin [IL]-1β; IL-6=interleukin [IL]-6; IL-8= interleukin [IL]-8; IL-18=interleukin [IL]-18; MCP-1=Monocyte chemoattractant protein-1; NETs= Neutrophil extracellular traps; TNF-α=tumor necrosis factor [TNF]-α.

In patients with BE-FAO, regardless of the ROSE status, similar alpha diversity and beta diversity were found for the lung microbiota communities, as depicted in **Figures 32A and 32B**. Crucially, alpha diversity in the BE-FAO ROSE (−) group was significantly lower than that in the COPD group ($P < 0.001$). Alpha diversity was similar

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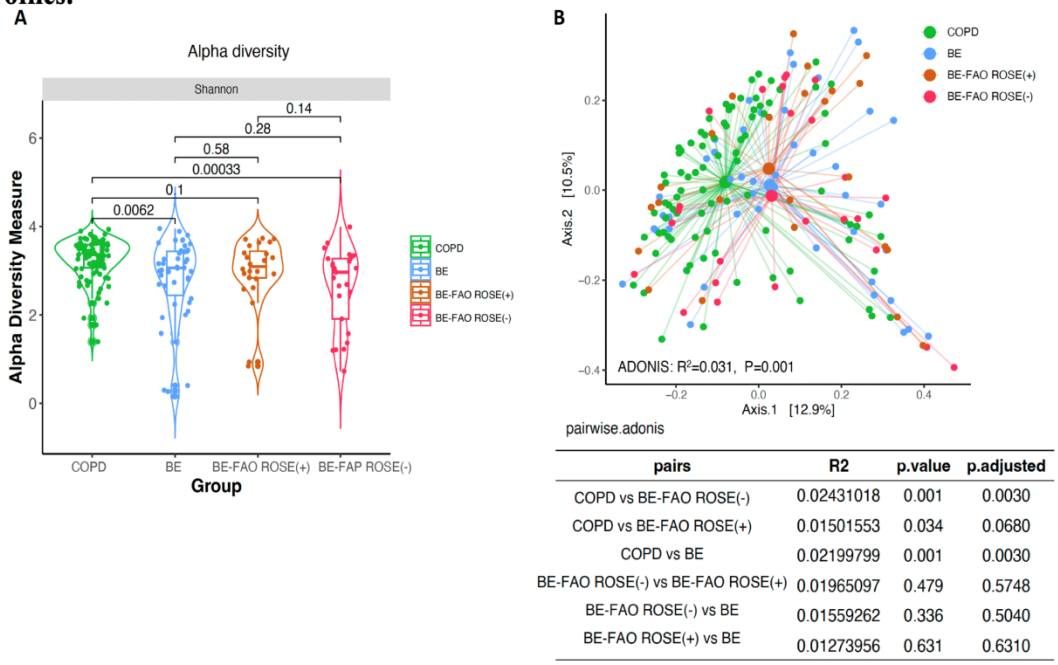
in the BE-FAO ROSE (+) group and the COPD group ($P = 0.1$). Furthermore, the pairwise analysis revealed marked differences in beta diversity between the COPD group and the BE-FAO ROSE (−) group (ADONIS PERMANOVA $R^2 = 0.024$, adjusted $P = 0.003$). However, these differences were less pronounced between the COPD group and the BE-FAO ROSE (+) group (ADONIS PERMANOVA $R^2 = 0.015$, $P = 0.034$, adjusted $P = 0.068$).

Figure 32 Alpha diversity (A) and beta diversity (B) of patients based on lung microbiome profiles.



pairwise.adonis

pairs	R2	p.value	p.adjusted
COPD vs BE-FAO ROSE(-)	0.02431018	0.001	0.0030
COPD vs BE-FAO ROSE(+)	0.01501553	0.034	0.0680
COPD vs BE	0.02199799	0.001	0.0030
BE-FAO ROSE(-) vs BE-FAO ROSE(+)	0.01965097	0.479	0.5748
BE-FAO ROSE(-) vs BE	0.01559262	0.336	0.5040
BE-FAO ROSE(+) vs BE	0.01273956	0.631	0.6310

Figure 32. Alpha diversity (A) and beta diversity (B) of patients based on lung microbiome profiles. (**A**) BE, BE-FAO ROSE (+), and BE-FAO ROSE (−) patients showed comparable alpha diversity levels. (**B**) Marked differences emerged in beta diversity between the BE-FAO ROSE (−) and COPD groups (adjusted P = 0.003). In contrast, the differences between BE-FAO ROSE (+) and COPD were less pronounced (adjusted P value = 0.068). BE=Bronchiectasis without fixed airflow obstruction, BE-FAO=Bronchiectasis with fixed airflow obstruction, COPD=Chronic obstructive pulmonary disease, ROSE=Radiology, Obstruction, Symptoms, Exposure

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The BE-FAO ROSE (+) group had a notably higher relative abundance of *Candidatus Absconditabacteria* (*P* = 0.034). The BE-FAO ROSE (−) group exhibited a slightly increased, but not statistically significant, abundance of *Pseudomonas aeruginosa* (*P* = 0.086) (**Figures 33 and 34**). After adjustment for age and gender, DESeq2 analysis revealed that the BE-FAO ROSE (−) group had higher levels of species such as *Pseudoleptotrichia goodfellowii* and *Streptococcus mutans* than the ROSE(+) group (**Figure 35**).

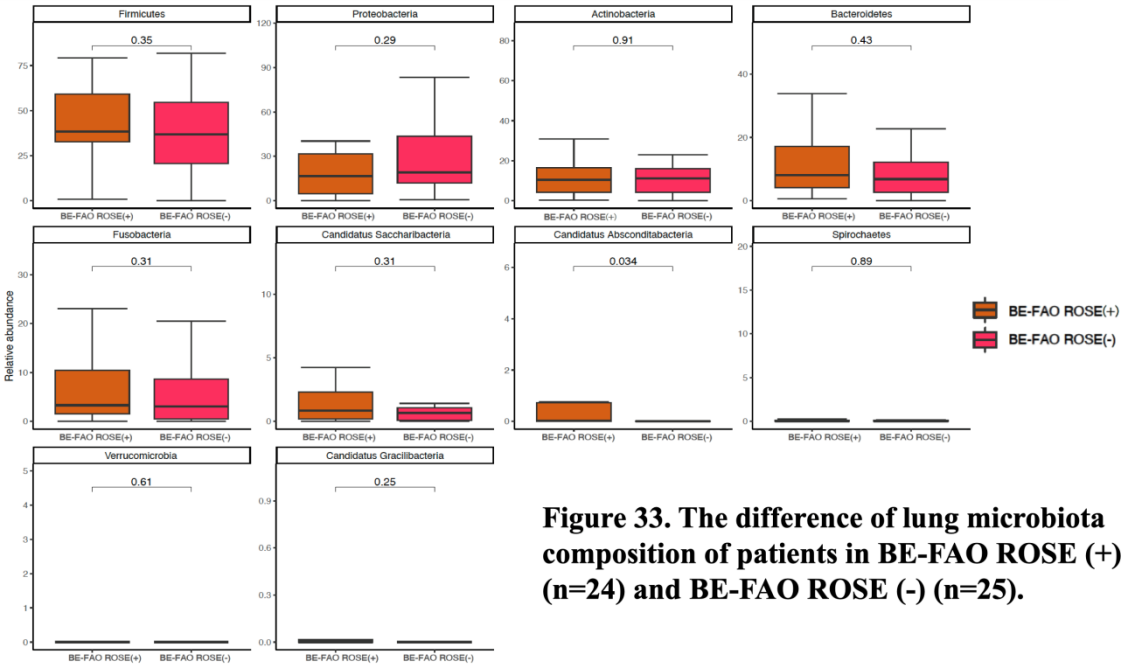

Figure 33. The difference of lung microbiota composition of patients in BE-FAO ROSE (+) (n=24) and BE-FAO ROSE (-) (n=25).

Figure 33. The difference of lung microbiota composition of patients in BE-FAO ROSE (+) (n=24) and BE-FAO ROSE (-) (n=25). The composition of major taxonomic groups and the distribution of relative abundance of phylum level. The patients with BE-FAO ROSE (+) had a higher relative abundance of *Candidatus Absconditabacteria* (*P*=0.034) at the phyla level compared to those with BE-FAO ROSE (-). BE-FAO= Bronchiectasis with fixed airflow obstruction; ROSE=Radiology, Obstruction, Symptoms, Exposure.

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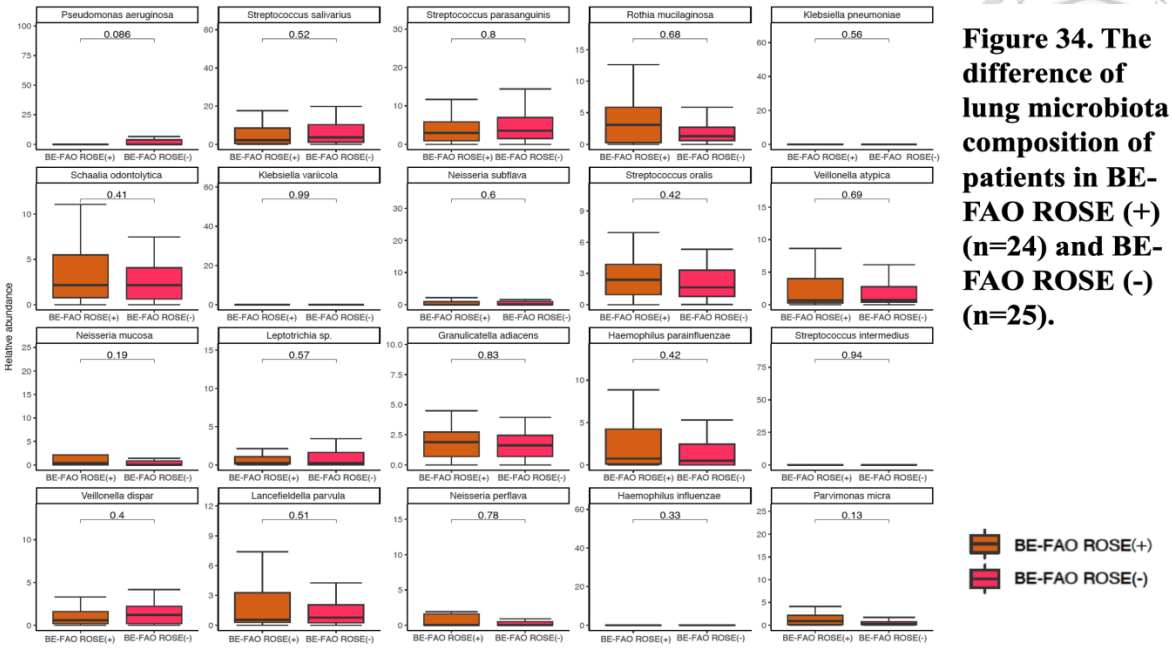

Figure 34. The difference of lung microbiota composition of patients in BE-FAO ROSE (+) (n=24) and BE-FAO ROSE (-) (n=25).

Figure 34. The difference of lung microbiota composition of patients in BE-FAO ROSE (+) (n=24) and BE-FAO ROSE (-) (n=25). The composition of major taxonomic groups and the distribution of relative abundance of species level. The patients with BE-FAO ROSE (-) had a relative abundance of *Pseudomonas aeruginosa* (*P*=0.086) when ASV annotated to species level, compared with those BE-FAO ROSE (+). BE-FAO= Bronchiectasis with fixed airflow obstruction; ROSE=Radiology, Obstruction, Symptoms, Exposure.

Figure 35

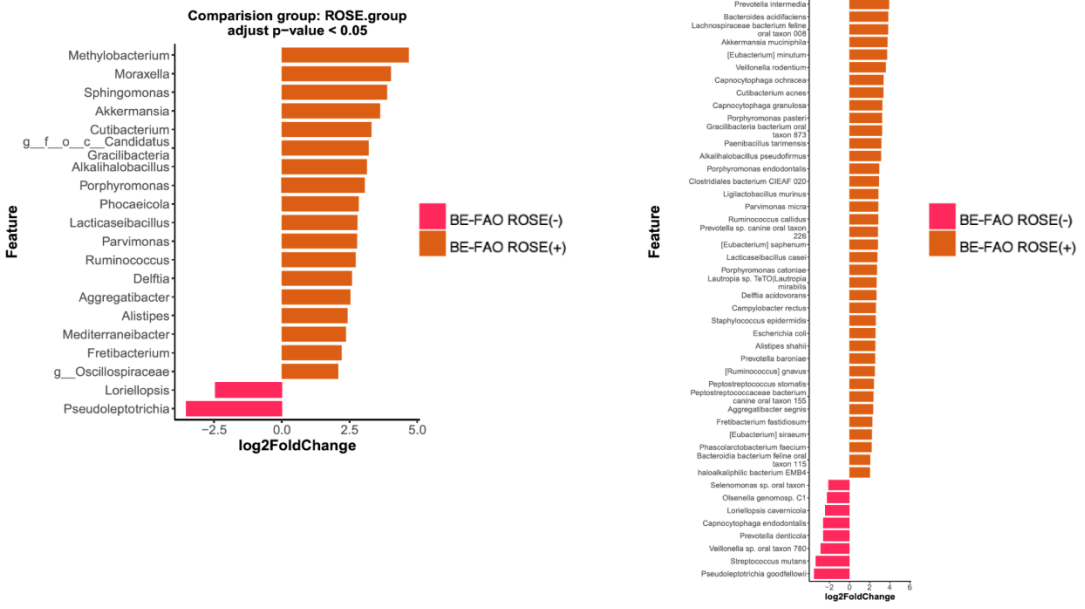

Figure 35. The differential abundance of lung microbiome analysis using DEseq2 (after adjusting for age and gender) in the BE-FAO group. The different taxonomic levels (adjusted p<0.05 and fold

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change>2.0) in BE-FAO ROSE (+) versus BE-FAO ROSE (-) at (A) genus level (B) species level. We further disclosed that *Pseudoleptotrichia goodfellowii*, *Streptococcus mutans, Veillonella sp.oral taxon 780, Prevotella denticola, Capnocytophaga endodontalis, Loriellopsis cavernicola, Olsenella genomosp.C1 and Selenomonas sp. oral taxon* were enriched in BE-FAO ROSE (-) group compared to BE-FAO ROSE (+) group. BE-FAO= Bronchiectasis with fixed airflow obstruction; ROSE=Radiology, Obstruction, Symptoms, Exposure.

3.6 Association of specific lung bacterial taxa and airway inflammation with risk of future exacerbations in BE-FAO

During a median follow-up of 2.46 years (range, 1.45–3.10), 47 participants (25.9% of those enrolled) experienced moderate-to-severe exacerbations, totaling 80 episodes. The BE-FAO group, including the ROSE (+) and ROSE (−) subgroups, had a significantly higher risk of exacerbations than the COPD and BE groups **(Figure 36)**.

Figure 36. Time to first moderate-severe exacerbation:

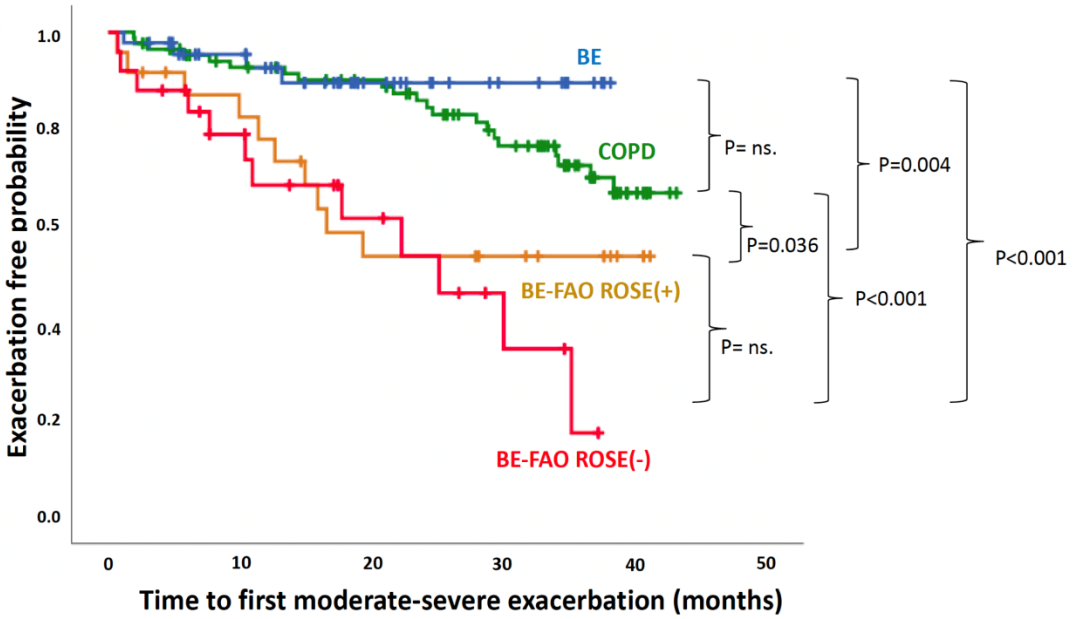

Figure 36. Time to first moderate-severe exacerbation. Comparing COPD, BE, and BE-FAO (Incorporating ROSE (+) and ROSE (-) subgroups). ns: not significant. BE=Bronchiectasis without fixed airflow obstruction; BE-FAO= Bronchiectasis with fixed airflow obstruction; COPD=Chronic obstructive pulmonary disease; ROSE=Radiology, Obstruction, Symptoms, Exposure.

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Clinically, as detailed in **Table 9**, patients with BE-FAO with a higher risk of exacerbations had higher blood neutrophil counts and levels of neutrophilic inflammatory cytokines (IL-1β and IL-8) in the BAL samples as well as lower FVC scores.

Table 9 Clinical variables of patients with bronchiectasis with FAO

	BE with FAO (n=49)		
Clinical factors/variables	**Non-exacerbation**	**Exacerbation**	*P* value
Number	**27**	**22**	
Age, years, median (IQR)	73.6(61.5-79.1)	73.4(63.8-78.9)	0.968
Gender, Man, n(%)	21(77.8)	14(63.3)	0.220
BMI (kg/m^2), median (IQR)	22.3(19.9-25.4)	21.6(19.4-23.5)	0.335
Smoking status, n (%)			
Nonsmoker	13 (48.1)	12(54.5)	0.437
Current smoker or ex-smoker	14(51.8)	10(45.5)	
Classification of BE-FAO			
ROSE criteria (+)	14(51.9)	10(45.5)	0.437
ROSE criteria (-)	13 (48.1)	12(54.5)	
History of tuberculosis infection, n (%)	7(25.9)	7(31.8)	0.444
Lung function test, median (IQR)			
FEV_1/FVC (%)	62.9(59.7-66.2)	64.8(57.5-67.5)	0.494
FEV_1 (%)	73.5(55.8-83.5)	64.9(50.4-72.4)	0.062
FVC (%)	91.1(78.6-108.5)	80.4(68.9-89.0)	0.017*
Bronchodilator reversibility, n (%)	1(2.7)	5(22.7)	0.056
Radiological severity of Bronchiectasis			
Bronchiectasis involved lobes, median (IQR)	4.0(3.0-5.0)	5.0(3.0-6.0)	0.361
Modified Reiff score, median (IQR)	4.0(3.0-6.0)	5.0(3.0-6.25)	0.422
Bronchiectasis severity index (BSI), median (IQR)	7.0(5.0-9.0)	9.0(7.0-10.2)	0.057
mild (0-4)	5(18.5)	1(4.5)	
moderate (5-8)	12(44.0)	8(36.4)	0.184
severe (≥9)	10(37.0)	13 (59.1)	
Emphysema LAV (%), median (IQR)	3.6(2.2-13.4)	5.6(3.7-17.6)	0.236
mMRC (dyspnea scale), n (%)			

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0-1	15(55.6)	9(40.9)	0.232
≥ 2	12(44.0)	13 (59.1)	
CAT score (symptoms score), n (%)			
<10	17(63.0)	18(81.8)	0.128
≥10	10(37.0)	4(18.2)	
Exacerbation in the prior year, n (%)			
High risk, ≥2 times/year	4(14.8)	7(31.8)	0.141
Low risk, 0-1 time/year	23(85.2)	15(68.2)	
Inhalation therapy, n(%) at baseline			
Short-acting bronchodilator or none	7(25.0)	3(13.6)	0.750
Monotherapy (LAMA or LABA)	2(7.4)	2(9.1)	
Dual bronchodilators (LAMA+LABA)	13(48.1)	13(59.1)	
Triple therapy	5(18.5)	4(18.2)	
Inhaled corticosteroid (ICS)	5(18.5)	4(18.2)	0.635
Blood sample, median (IQR)			
Hemoglobin (g/dl)	13.8(12.7-14.9)	13.8(12.7-14.7)	1.000
Platelet count (K/μl)	233.0(211.0-287.0)	254.0(200.2-286.2)	0.410
White blood cell counts (K cells/mm^3)	7.21(5.15-8.09)	7.60(5.42-9.40)	0.387
Neutrophil (%)	60.6(54.6-66.4)	69.3(63.1-74.0)	0.004*
Eosinophil (%)	2.8(1.9-4.4)	1.90(0.9-4.0)	0.086
Eosinophil counts (cells/mm^3)	182.9(136.3-289.5)	146.1(89.6-243.8)	0.240
CRP (mg/dL)	0.38(0.15-0.77)	0.73(0.27-1.15)	0.112
Bronchoalveolar lavage sample, median (IQR)			
Macrophage (%)	84.9(77.9-91.2)	86.9(81.2-92.0)	0.520
Neutrophils (%)	2.4(1.2-7.7)	2.1(0.6-4.6)	0.269
Eosinophils (%)	1.7(1.0-2.5)	2.1(1.3-4.0)	0.260
Lymphocyte %	8.4(3.9-10.7)	7.0(3.7-11.0)	0.630
Bronchoalveolar lavage sample, mean (SD)			
Eotaxin (pg/ml)	2.61(1.10-4.43)	1.75(0.87-2.44)	0.215
IL-1β (pg/ml)	39.7(4.02-265.1)	172.7(7.0-1210.0))	0.017*
IL-6 (pg/ml)	11.8(5.3-46.5)	43.4(19.0-105.4)	0.686
IL-18 (pg/ml)	36.2(23.4-52.5)	38.8(23.5-47.7)	0.618
IL-8 (pg/ml)	441.8(214.0-1535.3)	1557.7(312.5-3003.8)	0.017*
TNF-α (pg/ml)	12.7(4.8-30.1)	23.4(5.6-86.6)	0.063

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MCP-1(pg/ml)	251.4(118.6-542.4)	356.3(202.4-578.9)	0.949
NETs (pg/ml)	0.88(0.35-2.58)	1.65(0.63-2.95)	0.301
Conventional culture of BAL samples			
Klebsiella pneumoniae, n (%)	10(37.0)	8(36.4)	0.599
Pseudomonas aeroginosa, n (%)	7(25.9)	9(40.9)	0.210
Staphylococcus aureus, n (%)	6(22.2)	5(22.7)	0.616
Haemophilus influenzae, n (%)	1(3.7)	2(9.1)	0.422
Non-tuberculosis mycobacterium, n(%)	2(7.4)	5(22.7)	0.133
Asepergillus spp, n (%)	2(7.4)	3(13.6)	0.401
Candida spp, n (%)	8(29.6)	4(18.2)	0.279
Potential pathogenic bacteria colonization, n (%)	23(85.5)	21(95.5)	0.245

Data are presented as No. (%) or median (interquartile range), unless otherwise indicated; For each row, data are either % with *P*-values from *t* test or Fisher's exact tests between the two groups, median (IQR) with p-values from Mann-Whitney tests. BAL=Bronchoalveolar lavage; BE=Bronchiectasis without fixed airflow obstruction; BE-FAO=Bronchiectasis with fixed airflow obstruction; BMI=Body Mass Index; COPD=Chronic obstructive pulmonary disease; ROSE=Radiology, Obstruction, Symptoms, Exposure; FEV1=forced expiratory volume in 1 sec; FVC=forced vital capacity; LAV=low-attenuation volume; HU: Hounsfield unit; CAT=COPD Assessment Test; mMRC=modified Medical Research Council; IL-1β=interleukin [IL]-1β; IL-6=interleukin [IL]-6; IL-8= interleukin [IL]-8; IL-18=interleukin [IL]-18; MCP-1=Monocyte chemoattractant protein-1; NETs= Neutrophil extracellular traps; TNF-α=tumor necrosis factor [TNF]-α.

Results from the lung microbiome analysis revealed similar alpha diversity ($P = 0.12$) and beta diversity ($R^2 = 0.025$, $P = 0.24$) in the exacerbation and non-exacerbation subgroups of the BE-FAO group (**Figure 37**). Despite this similarity, the exacerbation subgroup tended to exhibit a higher relative abundance of *Proteobacteria* ($P = 0.075$), although the finding was nonsignificant **(Figure 38).** Further DESeq2 analysis identified a predominance of specific pathogens such as *Leptotrichia sp. canine oral taxon* 345, *Haemophilus parahaemolyticus, Pseudomonas aeruginosa, Bacteroides pyogenes*, and

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Tropheryma whipplei in the exacerbation subgroup relative to the non-exacerbation subgroup (**Figure 39**).

Figure 37 Alpha (A) and beta (B) diversity in BE-FAO patients

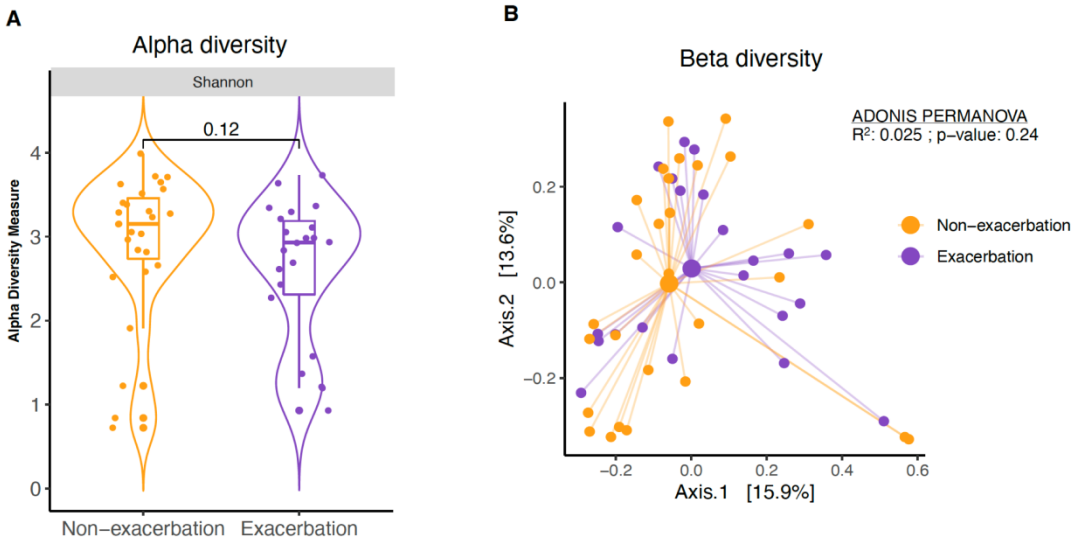

Figure 37 Alpha (A) and beta (B) diversity in BE-FAO patients with future exacerbations (n=22) versus those without (n=27) using BAL microbiome profiles. Both alpha diversity (P = 0.12) and beta diversity (R^2 = 0.025, P = 0.24) measures were similar between exacerbation and non-exacerbation subgroups. BE-FAO= Bronchiectasis with fixed airflow obstruction.

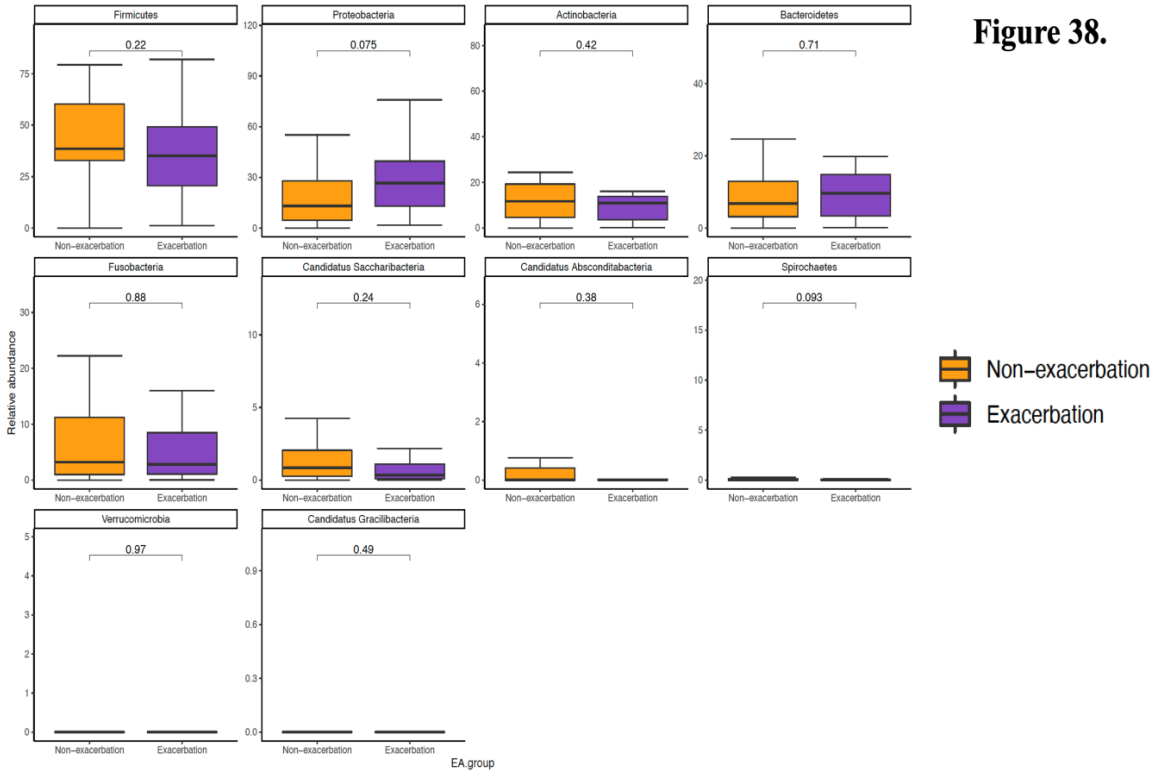

Figure 38.

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Figure 38. Differences in lung microbiota composition at the phylum level between patients with exacerbations (n = 22) and non-exacerbations (n = 27) in the BE-FAO group. In this group, the exacerbation subgroup had a higher relative abundance of *Proteobacteria* (*P* =0.075) compared with the non-exacerbation subgroup, although this difference was nonsignificant. No significant differences were obtained in other major phyla between the exacerbation and non-exacerbation subgroups. BE-FAO = bronchiectasis with FAO.

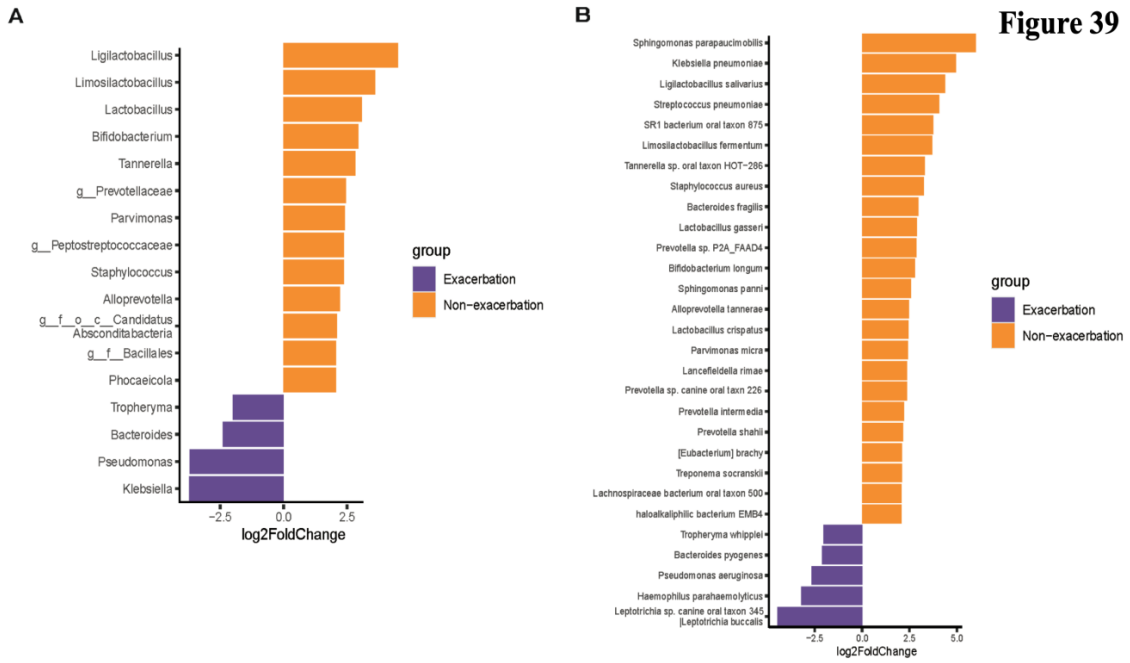

Figure 39. The differential abundance of lung microbiome analysis using DEseq2 in the BE-FAO group. The different taxonomic levels (adjusted p<0.05 and fold change>2.0) in exacerbation versus non-exacerbation subgroups at (A) phylum level (B) species level. DESeq2 analysis revealed that the exacerbation subgroup of BE-FAO had a predominance of *Leptotrichia sp. canine oral taxon 345, Haemophilus parahaemolyticus, Pseudomonas aeruginosa, Bacteroides pyogenes,* and *Tropheryma whipplei* relative to the non-exacerbation subgroup. BE-FAO= Bronchiectasis with fixed airflow obstruction.

Notably, two oral species—*Treponema socranskii* and *Dialister invisus*—were significantly correlated with higher levels of neutrophilic cytokines (BAL-IL 1β and BAL-IL 8), highlighting their potential role in the risk of exacerbations in the BE-FAO group (**Figure 40**).

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Figure 40. The correlation of clinical variables and lung microbiota in the bronchiectasis with FAO group.

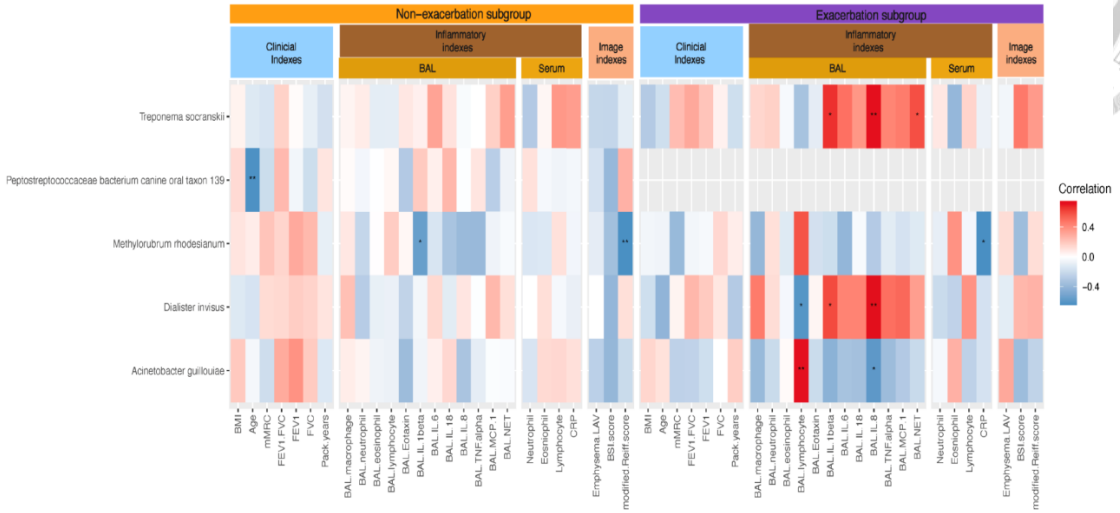

Figure 40. The correlation of clinical variables and lung microbiota in the bronchiectasis with FAO group. Heatmap showing spearman correlation between clinical variables and BAL microbiome in exacerbation subgroup and non-exacerbation subgroup. Clinical variables are grouped into three categories: clinical indexes, inflammatory indexes, and imaging indexes. Only those taxa that displayed at least one significant correlation ($q < .01$, following FDR correction) were selected. The color-coded matrix represents the Spearman correlation coefficient, with red indicating a positive correlation and blue indicating a negative correlation. FDRs are denoted: $*q < 0.05$; $**q < 0.01$; $***q < 0.001$. The spearmans correlation revealed two oral taxa, *Treponema socranskii* and and *Dialister invisus*, in exacerbation group of BE-FAO were positively associated neutrophilic cytokines (BAL-IL 1β and BAL-IL 8). BAL= Bronchoalveolar lavage; BE=Bronchiectasis without fixed airflow obstruction; BE-FAO=Bronchiectasis with fixed airflow obstruction; BMI=Body Mass Index; BSI=Bronchiectasis severity index; CAT=COPD Assessment Test; COPD= Chronic obstructive pulmonary disease; CRP=C-reactive protein; FDR=False discovery rate; FEV_1=forced expiratory volume in 1 sec; FVC=forced vital capacity; LAV=low-attenuation volume; mMRC=modified Medical Research Council; IL-1β=interleukin [IL]-1β; IL-6=interleukin [IL]-6; IL-8= interleukin [IL]-8; IL-18=interleukin [IL]-18; MCP-1=Monocyte chemoattractant protein-1; NETs= Neutrophil extracellular traps; TNF-α=tumor necrosis factor [TNF]-α.

3.7 The Impact of COPD-Bronchiectasis association on clinical outcomes: validation of the ROSE criteria in two cohorts

To further evaluate the clinical implications and outcomes of the COPD-BE association in East Asian populations, our study applied the ROSE criteria to assess the

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prevalence, clinical impact, and outcomes of the COPD-BE association [BE-FAO ROSE (+)] in two cohorts, comparing it with nonsmoking BE with fixed airflow obstruction (FAO) [BE-FAO ROSE (-)] and those without FAO (Bronchiectasis only). In addition to my prospective cohort, we also enrolled a multicenter retrospective cohort from 16 hospitals in the Taiwan Bronchiectasis Research Collaboration (TBARC) [114]. In my prospective cohort, patients with bronchiectasis were enrolled from November 2018 to December 2023 at the National Taiwan University Hospital (NTUH), Yunlin Branch. In the multicenter retrospective cohort, data from 2,753 adults aged 20 years and older, diagnosed with bronchiectasis according to the 2017 European Respiratory Society (ERS) guidelines [115], were collected between January 2017 and June 2020. Clinical data were gathered for one-year post-enrollment from patients who attended at least two follow-up visits at chest clinics.

3.7.1 Stratification of bronchiectasis in cohorts based on ROSE criteria

According to the ROSE criteria [76], which include Radiological evidence of bronchiectasis (R), Obstruction characterized by a post-bronchodilator FEV1/FVC ratio <0.7 (O), respiratory Symptoms (S), and Exposure exceeding a 10 pack-year smoking history (E), we objectively define the COPD-BE association along with other subgroups. Therefore, we could categorize the patients into four groups: nonsmoking BE (without airflow obstruction), smoking BE (without airflow obstruction), nonsmoking BE with

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FAO, and those who fully meet the ROSE criteria (COPD-BE association). This categorization facilitates further detailed analysis.

Figure 41

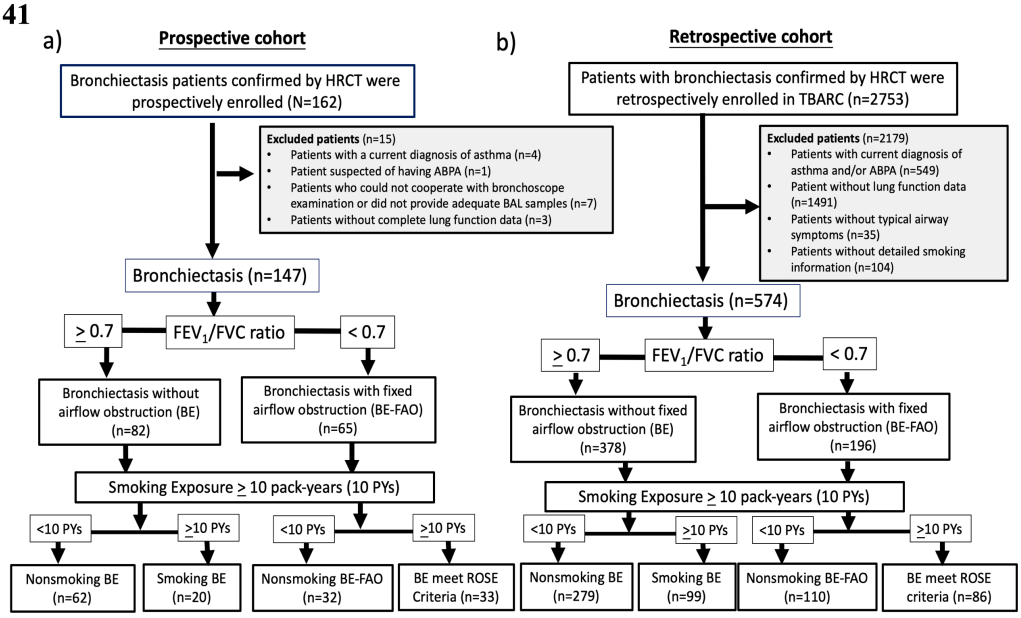

Figure 41. The workflow of patients recruited in the prospective cohort (A) and retrospective cohort (B). ABPA=Allergic bronchopulmonary aspergillosis; BE=Bronchiectasis; BE-FAO=Bronchiectasis with fixed airflow obstruction; COPD=Chronic obstructive pulmonary disease; FEV_1=forced expiratory volume in 1 sec; FVC=forced vital capacity; HRCT: high-resolution computed tomography, ROSE criteria: Radiological bronchiectasis(R), Obstruction defined by a post-bronchodilator FEV_1/FVC ratio <0.7 (O), Symptoms (S), and Exposure to a minimum of 10 pack-year smoking history (E); TBARC=Taiwan Bronchiectasis Research Collaboration

In the prospective cohort, 162 patients with bronchiectasis confirmed by HRCT were enrolled. Four patients had co-existing asthma, one was suspected of having ABPA, seven could not cooperate with the bronchoscopy examination or did not provide adequate BAL samples, and three lacked complete lung function data. Consequently, a total of 147 patients were included for further analysis (**Figure 41A**). In the retrospective cohort (n=2,753), 2,179 patients were excluded for the following reasons: asthma and/or

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ABPA (n=549), absence of lung function data (n=1,491), lack of typical airway symptoms (n=35), and incomplete smoking history (n=104). Ultimately, 574 patients were included for further analysis (**Figure 41B**). Based on the ROSE criteria for lung function and smoking exposure, the prospective cohort was divided into four subgroups: nonsmoking BE (n=62), smoking BE (n=20), nonsmoking BE with FAO (n=32), and BE meeting the ROSE criteria (n=33). Similarly, in the retrospective cohort, the subgroups included nonsmoking BE (n=279), smoking BE (n=99), nonsmoking BE with FAO (n=110), and BE meeting the ROSE criteria (n=86) **(Figure 41**).

3.7.2 Clinical characteristics of COPD-BE association and other groups in the prospective cohort

Table 10 summarizes the demographics of the prospective cohort. The COPD-BE association group and the nonsmoking BE with FAO group comprise 22.4% and 21.7% of the cohort, respectively, and consist mainly of older individuals (median age 73.9 years and 71.3 years, respectively) compared to the nonsmoking BE group. However, the COPD-BE association group is primarily male, whereas the nonsmoking BE with FAO group is predominantly female (59.4%). Notably, the lung function and radiologic scores of both the COPD-BE association and nonsmoking BE with FAO groups are similar. Clinically, these groups exhibit a high prevalence of COPD diagnosis (66.7% and 59.4%, respectively), significantly higher than in other subgroups. As expected, these patients

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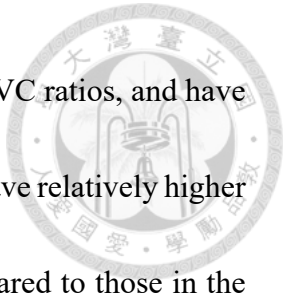

also demonstrate poorer lung function, with lower FEV_1 and FEV_1/FVC ratios, and have the highest BSI scores compared to other groups. Additionally, they have relatively higher modified Reiff scores and more bronchiectasis-involved lobes compared to those in the nonsmoking BE without FAO group. A comparison of the radiological locations of bronchiectasis revealed that the COPD-BE association group is more likely to have bronchiectasis affecting both the upper and lower lobes.

Table 10. Stratification of demographic details in bronchiectasis patients by ROSE criteria (prospective Cohort, N=147)

Variables	Nonsmoking BE	Smoking BE	Nonsmoking BE with FAO	COPD-BE association	P value
Number, (%)	**62 (42.1)**	**20 (13.6)**	**32 (21.7)**	**33 (22.4)**	
Demographics					
Age, years, median (IQR)	63.4(54.9-73.1)	71.7(62.7-78.1)	71.3(63.0-79.5)	73.9(63.9-78.6)	0.001*[ad]
Gender, Male, n (%)	21(33.9)	17(85.0)	13(40.6)	33(100)	<0.001*
BMI, median (IQR)	20.2(18.1-22.5)	23.0(20.2-26.5)	21.9(19.5-24.4)	21.9(20.0-25.4)	0.051
Nonsmoker	60(96.8)	0(0)	28(84.8)	0(0)	<0.001*
Ex-or current smokers	2(3.2)	20(100)	4(12.5)	33(100)	
Clinical status					
CAT score, median (IQR)	6.0(3.0-12.0)	10(2.2-16.0)	6.0(3.2-8.7)	9.0(3.5-12.0)	0.696
mMRC, median (IQR)	1(0-1)	2(1-2)	1(1-2)	2.0(1.0-2.0)	<0.001*[adf]
Hospitalization in prior year, n(%)	8(12.9)	4(20.0)	8(25.0)	9(27.3)	0.312
Comorbidities, n (%)					
Cardiovascular disease	21(33.9)	5(25.0)	13(40.6)	13(29.4)	0.654
Stroke	2(3.2)	1(5.0)	0(0)	3(9.1)	0.304
Hyperlipidemia	5(8.1)	5(25.0)	2(6.3)	5(15.2)	0.134
Diabetes Mellitus	4(6.5)	5(25.0)	3(9.4)	4(12.1)	0.139
Old tuberculosis infection	13(21.0)	6(30.0)	14(43.8)	7(21.2)	0.097
COPD	7(11.3)	8(40.0)	19(59.4)	22(66.7)	<0.001*
Chronic kidney disease	6(9.7)	4(20.0)	5(15.6)	5(15.2)	0.640

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Depression	2(3.2)	1(5.0)	1(3.0)	0(0)	0.707
Autoimmune disease	7(11.3)	2(10.0)	1(3.1)	1(3.0)	0.347
Reflux disease	31(50.0)	7(35.0)	14(43.8)	12(36.4)	0.505
Rhinosinusitis	2(3.2)	0(0)	3(9.4)	1(3.0)	0.341
Allergic rhinitis	30(48.4)	6(30.0)	12(37.5)	13(39.4)	0.462
Lung Functions					
FEV_1/FVC (%)	78.5(74.9-81.2)	77.8(73.7-80.8)	64.7(61.0-68.3)	62.3(49.9-66.2)	<0.001*[abde]
FEV_1 (%)	88.9(72.6-101.0)	96.7(81.3-103.5)	71.8(58.8-80.5)	70.0(43.6-76.4)	<0.001* [abde]
FEV_1,L	2.0(1.4-2.4)	2.1(1.8-2.7)	1.5(1.1-1.7)	1.6(1.1-1.9)	<0.001*[abde]
FVC (%)	94.0(79.3-102.9)	91.2(82.0-103.5)	88.9y76(75.2-100.3)	87.8(75.9-98.7)	0.632
FVC, L	2.6(1.9-3.0)	2.6(2.3-3.5)	2.2(1.8-2.7)	2.7(2.2-3.3)	0.032*
Severity score, median (IQR)					
BSI score	6.0(3.0-9.0)	6.0(4.5-12.2)	9.0(6.2-12.0)	9.0(6.0-12.0)	0.002*[ad]
E-FACED	1.0(0-3.0)	3.0(1.0-4.5)	3.0(2.0-4.0)	3.0(2.0-5.0)	<0.001*[ad]
Modified Reiff score	4.0(2.0-5.0)	3.0(2.0-4.7)	5.0(3.0-7.0)	5.0(3.0-6.0)	0.041*
Involved lobe numbers	3.0(2.0-4.0)	3.0(2.0-4.0)	4.0(3.0-5.0)	4.0(2.5-5.5)	0.005*[ad]
RUL, n(%)	26(41.9)	9(45.0)	25(78.1)	25(75.8)	0.001*
RML, n%)	44(71.0)	14(70.0)	24(75.0)	21(63.6)	0.790
RLL, n(%)	35(56.5)	11(55.0)	18(56.3)	26(78.8)	0.136
LUL, n(%)	18(29.0)	6(30.0)	16(50.0)	14(42.4)	0.183
Left lingular lobe, n(%)	34(54.8)	11(55.0)	19(59.4)	19(57.6)	0.976
LLL, n(%)	27(43.5)	9(45.0)	23(71.9)	25(75.8)	0.004*

Data are presented as No. (%) or median (interquartile range), unless otherwise indicated; n.a.: not available. For each row, data are either presented as % with p-values from t-tests or Fisher's exact tests between the groups or as median (IQR) with p-values from Kruskal-Wallis tests. *p < 0.05.

Bonferroni correction was applied for statistically significant variables, with comparisons between the following groups:

a. COPD-BE association versus nonsmoking BE, p<0.05

b. COPD-BE association versus smoking BE, p<0.05

c. COPD-BE association versus nonsmoking BE with FAO, p<0.05

d. Nonsmoking BE with FAO versus nonsmoking BE, p<0.05

e. Nonsmoking BE with FAO versus smoking BE, p<0.05

f. Smoking BE versus nonsmoking BE, p<0.05

Abbreviations: BE = Bronchiectasis; BMI = Body Mass Index; BSI=Bronchiectasis severity index; CAT = COPD Assessment Test; COPD = Chronic obstructive pulmonary disease; E-FACED=Exacerbation,

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forced expiratory volume in 1s (FEV_1), age, chronic colonization by *Pseudomonas aeruginosa*, radiological **e**xtension and **d**yspnea; FAO=Fixed airflow obstruction; FEV_1 = forced expiratory volume in 1 sec; FVC= forced vital capacity; LUL=Left upper lobe; LLL=Left lower lobe; mMRC =modified Medical Research Council; ROSE=Radiology, Obstruction, Symptoms, Exposure; RUL=Right upper lobe; RML=Right middle lobe; RLL=Right lower lobe.

Table 11 reveals that the COPD-BE association group and nonsmoking BE with FAO had similar blood and BAL immune cell profiles, as well as inflammatory markers (**Figure 42**). Notably, both the COPD-BE association and nonsmoking BE with FAO groups had significantly higher neutrophil and monocyte counts, as well as elevated C-reactive protein (CRP) levels, compared to the nonsmoking BE patients. Patients in the COPD-BE association group also had higher blood eosinophil levels than those in the nonsmoking BE group ($p<0.05$) (**Table 11**, **Figure 42**). Microbiologically, *Klebsiella pneumoniae* was notably more prevalent in both the COPD-BE association and smoking BE groups ($p=0.021$), while *Staphylococcus aureus* was more commonly found in patients with nonsmoking BE with FAO ($p=0.012$). Regarding treatment, the COPD-BE association and nonsmoking BE with FAO groups were more frequently prescribed combination inhaler therapies, including dual bronchodilators, and utilized triple therapy more extensively than other subgroups.

Table 11. Comparison of laboratory results, treatment, and outcomes of patients stratified by ROSE criteria (prospective cohort, N=147)

Variables	Nonsmoking BE	Smoking BE	Nonsmoking BE with FAO	COPD-BE association	P value
Number, (%)	62 (42.1)	20 (13.6)	32(21.7)	33 (22.4)	

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Blood samples, median (IQR)					
Hemoglobulin (g/dL)	13.7(12.7-14.5)	13.0(11.6-14.5)	13.2(12.3-14.6)	14.2(13.0-14.6)	0.263
Platelet (k/μL)	223(194-282)	228(167-300)	241(205-285)	243(207-291)	0.536
White blood cell (cells/mm^3)	5750(4840-7140)	7600(6175-8767)	7380(5380-8870)	7500(5400-9075)	0.005*adf
Neutrophils counts (cells/mm^3)	3578(2615-4456)	4416(3224-6134)	4523(3079-6064)	4240(3240-6284)	0.023*adf
Eosinophil counts (cells/mm^3)	105(59-212)	139(94-279)	156(122-225)	168(110-313)	0.025*a
Eosinophilic ≥300 cells/mm^3, n (%)	5(8.1)	4(20.0)	4(12.5)	9(27.3)	0.079
Lymphocyte counts (cells/mm^3)	1694(1289-2038)	1865(1515-2498)	1776(1556-2306)	1667(1177-1973)	0.162
Neutrophil-Lymphocyte ratio	2.1(1.4-3.1)	2.2(1.4-3.8)	2.2(1.6-3.5)	3.1(2.0-4.3)	0.058
Monocyte counts (cells/mm^3)	333(267-410)	450(365-548)	418(347-529)	482(339-607)	<0.001*adf
CRP (mg/dL)	0.12(0.04-0.32)	0.42(0.11-0.89)	0.41(0.14-0.82)	0.36(0.12-0.92)	0.004*adf
BAL samples, median (IQR)					
BAL Macrophage (%)	88.5(77.7-91.6)	85.2(74.9-89.8)	86.3(83.8-93.4)	87.7(82.4-91.9)	0.346
BAL Neutrophil (%)	1.1(0.6-3.0)	2.2(0.9-4.8)	1.93(0.8-5.3)	2.0(0.8-3.3)	0.167
BAL Eosinophil (%)	2.1(1.0-3.5)	2.5(1.1-5.2)	1.9(1.4-3.1)	2.4(1.2-4.1)	0.849
BAL Lymphocyte (%)	7.2(2.7-15.6)	7.3(3.8-15.3)	4.6(2.2-9.0)	7.2(4.4-10.0)	0.228
BAL conventional culture					
Klebsiella pneumoniae, n(%)	15(24.2)	11(55.0)	7(21.9)	14(42.4)	0.021*
Pseudomonas aeroginosa, n (%)	16(25.8)	5(25.0)	13(40.6)	8(24.2)	0.401
Staphylococcus aureus, n(%)	12(19.4)	0(0)	11(34.4)	4(12.1)	0.012*
Haemophilus influenzae, n (%)	8(12.9)	1(5.0)	2(6.3)	1(3.0)	0.328
NTM, n(%)	11(17.7)	0(0)	7(21.9)	4(12.1)	0.151
Treatment, n (%)					
Monotherapy	7(11.3)	4(20.0)	4(12.5)	2(6.1)	0.493
LAMA+LABA	13(21.0)	7(35.0)	19(59.4)	21(63.6)	<0.001*
Triple therapy	0(0)	1(5.0)	5(15.6)	6(18.2)	0.006*
Inhaled corticosteroid	0(0)	1(5.0)	5(15.6)	6(18.2)	0.006*
Macrolides	10(16.1)	3(15.0)	5(15.6)	7(21.2)	0.908

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Inhaled antibiotics	1(1.6)	1(5.0)	2(6.3)	2(6.1)	0.632
Systemic corticosteroids for exacerbation events	0(0)	1(5)	4(12.5)	9(27.3)	0.001*
Clinical outcomes					
Exacerbations	13(21.0)	5(25.0)	15(46.9)	17(51.5)	0.006*
Hospitalizations	6(9.7)	5(25.0)	6(18.8)	14(42.4)	0.003*
Mortality	1(1.6)	2(10.0)	1(3.1)	6(18.2)	0.016*

Data are presented as No. (%) or median (interquartile range), unless otherwise indicated

For each row, data are either presented as % with p-values from t-tests or Fisher's exact tests between the groups or as median (IQR) with p-values from Kruskal-Wallis tests. *p < 0.05.

Bonferroni correction was applied for statistically significant variables, with comparisons between the following groups:

a. COPD-BE association versus nonsmoking BE, p<0.05

b. COPD-BE association versus smoking BE, p<0.05

c. COPD-BE association versus nonsmoking BE with FAO, p<0.05

d. Nonsmoking BE with FAO versus nonsmoking BE, p<0.05

e. Nonsmoking BE with FAO versus smoking BE, p<0.05

f. Smoking BE versus nonsmoking BE, p<0.05

Abbreviations: BAL = Bronchoalveolar lavage; BE = Bronchiectasis; COPD = Chronic obstructive pulmonary disease; CRP= C-reactive protein; FAO=Fixed airflow obstruction; LABA = Long-acting β2 Sympathomimetic Agonists; LAMA = long-acting muscarinic antagonist; NTM= Non-tuberculosis mycobacteria; ROSE=Radiology, Obstruction, Symptoms, Exposure.

Figure 42

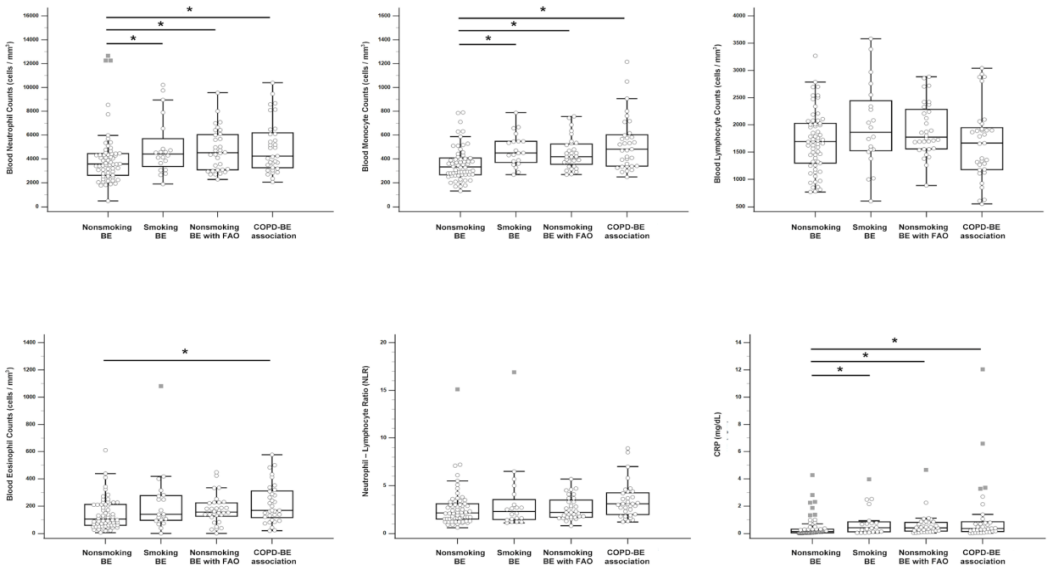

Figure 42. Box plots compare various blood biomarkers (neutrophils, eosinophils, lymphocytes, monocytes, NLR, and CRP levels) among four groups: nonsmoking bronchiectasis (BE), smoking BE,

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nonsmoking BE with fixed airflow obstruction (FAO), and COPD-BE association.
Significant differences ($p<0.05$) are indicated by asterisks (*). COPD=Chronic obstructive pulmonary disease; NLR: neutrophil-lymphocyte ratio; CRP=C-reactive protein (CRP) levels.

3.7.3 Clinical characteristics of COPD-BE association and other groups in the retrospective cohort

Table 12 reveals that the COPD-BE association cohort comprises 14.9% of the participants, while the nonsmoking BE with FAO group accounts for 19.1% of the cohort. The COPD-BE association group is predominantly male (96.5%), whereas the nonsmoking BE with FAO group is predominantly female (52.7%). This prevalence and gender difference between the two cohorts echoes the findings from the prospective cohort. Both the COPD-BE association and nonsmoking BE with FAO subgroups have a significantly higher prevalence of dyspnea (73.3% and 71.8%, respectively) compared to other subgroups. As expected, these groups also show a higher prevalence of COPD diagnoses (88.4% and 80.9%, respectively) and exhibit significantly lower lung function, as indicated by FEV_1/FVC ratios, mirroring the poorer lung function observed in Table 1. Notably, patients with nonsmoking BE with FAO exhibit significantly higher modified Reiff scores and a greater number of bronchiectasis-involved lobes, as well as lower FEV_1 and FVC levels, compared to those in the COPD-BE association group. Additionally, nonsmoking groups are more likely to have bronchiectasis affecting the right middle lobe and left lingular lobe compared to the smoking BE and COPD-BE association groups.

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Table 12. Clinical variables for bronchiectasis patients stratified by ROSE criteria (retrospective cohort, N=574)

Variables	Nonsmoking BE	Smoking BE	Nonsmoking BE with FAO	COPD-BE association	P value
Number, (%)	**279 (48.6)**	**99 (17.2)**	**110 (19.1)**	**86 (14.9)**	
Demographics					
Age, years, median (IQR))	68.2(61.3-76.0)	69.1(60.3-74.8)	69.4(61.2-77.8)	73.0(66.7-78.8)	0.001*[ab]
Gender, Male, n (%)	89(31.9)	88(88.9)	52(47.3)	83(96.5)	<0.001*
BMI, median (IQR)	20.5(18.5-23.0)	23.6(20.3-26.5)	21.0(18.6-23.8)	22.5(19.9-25.0)	<0.001*[aef]
Nonsmoker	253(90.7)	0(0)	87(79.1)	0(0)	<0.001*
Ex-or current smokers	26(9.3)	99(100%)	23(20.9)	86(100)	
Clinical status, n (%)					
Cough	251(90.0)	85(95.9)	102(92.7)	77(89.5)	0.440
Phlegm	226(81.0)	84(84.8)	98(89.1)	64(74.4)	0.048*
Hemoptysis	82(29.4)	15(15.2)	25(22.7)	14(16.3)	0.009*
Dyspnea	123(44.1)	55(55.6)	79(71.8)	63(73.3)	<0.001*
Comorbidities, n (%)					
Cardiovascular disease	68(24.4)	33(33.3)	33(30.0)	28(32.6)	0.232
Diabetes Mellitus	38(13.6)	30(30.3)	18(16.4)	19(22.1)	0.002*
Chronic kidney disease	24(8.6)	13(13.1)	15(13.6)	8(9.3)	0.378
Depression	15(5.4)	5(5.1)	2(1.8)	4(4.7)	0.494
COPD	92(33.0)	56(56.6)	89(80.9)	76(88.4)	<0.001*
Old tuberculosis infection	55(19.7)	15(15.2)	25(22.7)	18(20.9)	0.569
Autoimmune disease	11(3.9)	4(4.0)	3(2.7)	2(2.3)	0.850
Reflux disease	46(16.5)	24(24.2)	26(23.6)	10(11.6)	0.056
Rhinosinusitis	17(6.1)	3(3.0)	7(6.4)	5(5.8)	0.682
Lung Functions, median (IQR)					
FEV_1/FVC (%)	80.3(75.6-87.1)	77.6(74.7-83.7)	63.1(53.2-67.0)	56.4(49.1-63.4)	<0.001*[abde]
FEV_1,L	1.57(1.18-1.91)	1.99(1.56-2.53)	1.07(0.82-1.40)	1.32(1.02-1.80)	<0.001*[bcdef]
FVC, L	1.92(1.44-2.38)	2.51(1.90-3.26)	1.90(1.37-2.34)	2.45(2.08-3.00)	<0.001*[acef]
Radiological severity score, median (IQR)					
Modified Reiff score	5.0(3.0-8.0)	4.0(2.0-6.0)	6.0(3.0-10.0)	4.0(2.0-6.0)	<0.001*[ace]

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Involved lobe numbers	4.0(2.0-6.0)	3.0(2.0-5.0)	4.0(3.0-6.0)	3.0(2.0-4.0)	0.042*[c]
RUL, n(%)	168(60.2)	50(50.0)	71(64.5)	45(52.3)	0.116
RML, n%)	217(77.8)	69(69.7)	82(74.5)	50(58.1)	0.004*
RLL, n(%)	191(68.5)	68(68.7)	82(74.5)	68(79.1)	0.21
LUL, n(%)	136(48.7)	41(41.4)	55(50.0)	40(46.5)	0.583
Left lingular, n(%)	163(58.4)	44(44.4)	69(62.7)	37(43.0)	0.004*
LLL, n(%)	185(66.3)	67(67.7)	84(76.4)	51(53.9)	0.08

Data are presented as No. (%) or median (interquartile range), unless otherwise indicated

For each row, data are either presented as % with p-values from t-tests or Fisher's exact tests between the groups or as median (IQR) with p-values from Kruskal-Wallis tests. *p < 0.05.

Bonferroni correction was applied for statistically significant variables, with comparisons between the following groups:

a. COPD-BE association versus nonsmoking BE, p<0.05

b. COPD-BE association versus smoking BE, p<0.05

c. COPD-BE association versus nonsmoking BE with FAO, p<0.05

d. Nonsmoking BE with FAO versus nonsmoking BE, p<0.05

e. Nonsmoking BE with FAO versus smoking BE, p<0.05

f. Smoking BE versus nonsmoking BE, p<0.05

Abbreviations: BE = Bronchiectasis; BMI = Body Mass Index; COPD = Chronic obstructive pulmonary disease; FAO=Fixed airflow obstruction; FEV_1 = forced expiratory volume in 1 sec; FVC= forced vital capacity; LUL= Left upper lobe; LLL= Left lower lobe; ROSE=Radiology, Obstruction, Symptoms, Exposure; RUL=Right upper lobe; RML=Right middle lobe; RLL=Right lower lobe.

Table 13 demonstrates that the prevalence of *Pseudomonas aeruginosa* is significantly higher in the nonsmoking bronchiectasis with FAO group (p=0.002) compared to other groups. This pattern is similar in the prospective cohort, although it did not reach statistical significance. Conversely, while the prevalence of *Klebsiella pneumoniae* is higher in the COPD-BE association group, this increase is not statistically significant (p=0.066), suggesting a trend towards increased *Klebsiella pneumoniae* colonization in COPD-BE association patients, consistent with the prospective cohort. Regarding treatment, the utilization of dual bronchodilators and ICS-containing therapy

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is significantly greater in the COPD-BE association and nonsmoking bronchiectasis with FAO groups, reflecting previously noted trends towards more intensive treatment regimens in patients with FAO.

Table 13. Comparison of laboratory results, treatment and outcomes of patients stratified by ROSE criteria in retrospective cohort (N=574)

Variables	Nonsmoking BE	Smoking BE	Nonsmoking BE with FAO	COPD-BE association	P value
Number, (%)	**279 (48.6)**	**99 (17.2)**	**110 (19.1)**	**86 (14.9)**	
BAL conventional culture					
Klebsiella pneumoniae, n(%)	7(2.5)	4(4.0)	8(7.3)	7(8.1)	0.066
Pseudomonas aeroginosa, n (%)	15(5.4)	5(5.1)	18(16.4)	6(7.0)	0.002*
Staphylococcus aureus, n(%)	4(1.4)	0(0)	3(2.7)	1(1.2)	0.414
Haemophilus influenzae, n(%)	5(1.8)	1(1.0)	3(2.7)	0(0)	0.454
NTM, n(%)	41(14.7)	7(7.1)	15(13.6)	10(11.6)	0.261
Fungal colonization, n(%)	16(5.7)	9(9.1)	10(9.1)	3(3.5)	0.286
Treatment, n(%)					
Monotherapy	38(13.6)	15(15.2)	18(16.4)	16(18.6)	0.698
LAMA+LABA	53(19.0)	20(20.2)	48(43.6)	42(48.8)	<0.001*
ICS/LABA	13(4.7)	6(6.1)	9(8.2)	3(3.5)	0.446
Triple therapy	4(1.4)	10(10.1)	14(12.7)	15(17.4)	<0.001*
Inhaled corticosteroid	17(6.1)	15(15.2)	23(20.9)	18(20.9)	<0.001*
Macrolides	32(11.5)	11(11.1)	13(11.8)	7(8.1)	0.835
Inhaled antibiotics	2(0.7)	0(0)	0(0)	0(0)	0.547
Any oral antibiotics	17(6.1)	6(6.1)	7(6.4)	6(7.0)	0.992
Clinical outcomes					
Exacerbations	37(13.3)	11(11.1)	23(20.9)	22(25.6)	0.012*
Hospitalizations	48(17.2)	15(15.2)	27(24.5)	28(32.6)	0.006*
Mortality	3(1.1)	8(8.1)	2(1.8)	9(10.5)	<0.001*

Data are presented as No. (%) or median (interquartile range), unless otherwise indicated

For each row, data are either presented as % with p-values from t-tests or Fisher's exact tests between the

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groups

or as median (IQR) with p-values from Kruskal-Wallis tests. *p < 0.05.

Abbreviations: BE = Bronchiectasis; COPD = Chronic obstructive pulmonary disease; FAO=Fixed airflow obstruction; ICS= Inhaled corticosteroid; LABA = Long-acting β2 Sympathomimetic Agonists; LAMA = long-acting muscarinic antagonist; NTM= Non-tuberculosis mycobacteria; ROSE=Radiology, Obstruction, Symptoms, Exposure.

3.7.4 Clinical outcomes of COPD-BE association and other groups in the prospective cohort

In the prospective cohort, the median follow-up duration was 2.8 years (IQR: 1.6–4.2 years). **Figure 43A** shows that the COPD-BE association group and the nonsmoking BE with FAO group had significantly shorter time to the first exacerbation compared to the nonsmoking BE group (p=0.013 and p=0.037, respectively). **Figure 43B** further demonstrates that the COPD-BE association group had a significantly shorter time to the first hospitalization for exacerbation compared to both the nonsmoking BE (p=0.001) and nonsmoking BE with FAO (p=0.024) groups. These results are consistent with the patterns in **Table 11**. Additionally, we clarify that patients with COPD-BE association and nonsmoking BE with FAO had a higher risk of subsequent exacerbations (incidence rate ratio [IRR]: 3.68, 95% Confidence Interval [CI]: 2.32–5.86, and IRR: 2.37, 95% CI: 1.42–3.95, respectively) compared to those with bronchiectasis without FAO during follow-up (**Figure 44A**). Notably, in addition to antibiotics exposures, around 27.3% of patients in the COPD-BE association group who experienced exacerbations were exposed to systemic corticosteroids, a rate higher than that in other subgroups (**Table 11**).

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Figure 43

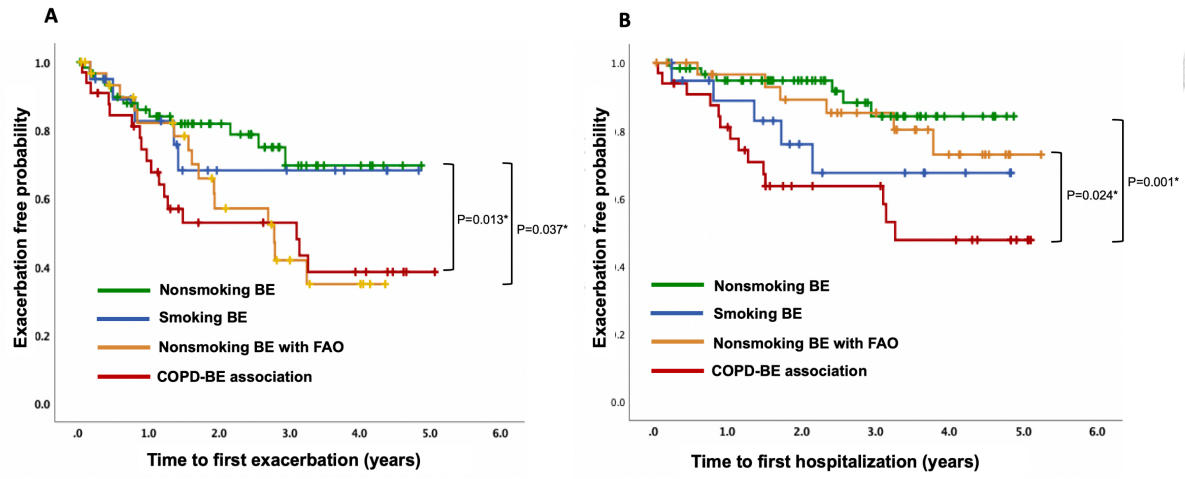

Figure 43. The clinical outcomes of four study subgroups in the prospective cohort. (A) the Kaplan-Meier plot illustrates the time to the first exacerbation among different bronchiectasis subgroups with a median follow-up of 2.8 years (interquartile range [IQR]: 1.6 to 4.2 years). The plot compares four groups: nonsmoking bronchiectasis (BE), smoking BE, nonsmoking BE with fixed airflow obstruction (FAO), and chronic obstructive pulmonary disease (COPD)-BE association. (B) the Kaplan-Meier plot illustrates the time to first hospitalization among different bronchiectasis subgroups with a median follow-up of 2.8 years (IQR: 1.6 to 4.2 years). The groups analyzed include nonsmoking BE, smoking BE, nonsmoking BE with FAO and the COPD-BE association.

The poor clinical outcomes in bronchiectasis are likely multifactorial, involving a combination of factors beyond smoking and lung function. Our logistic regression model accounted for these by including key variables such as age, sex, diabetes mellitus, cardiovascular disease, reflux disease, previous TB infection, *Pseudomonas aeruginosa, Klebsiella pneumoniae*, modified Reiff score, inhaled corticosteroids, and macrolide use. Even after these adjustments, patients with COPD-BE association and nonsmoking BE with FAO still exhibited a higher risk of exacerbations (adjusted Odds Ratio [aOR]: 4.06, 95% CI: 1.03–15.92 and aOR: 3.15, 95% CI: 1.02–9.67, respectively) compared to those

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without FAO. However, the risk of hospitalization was only significant in the COPD-BE association group after adjustment (aOR: 6.42, 95% CI: 1.16–35.48) (**Table 14**).

Figure 44

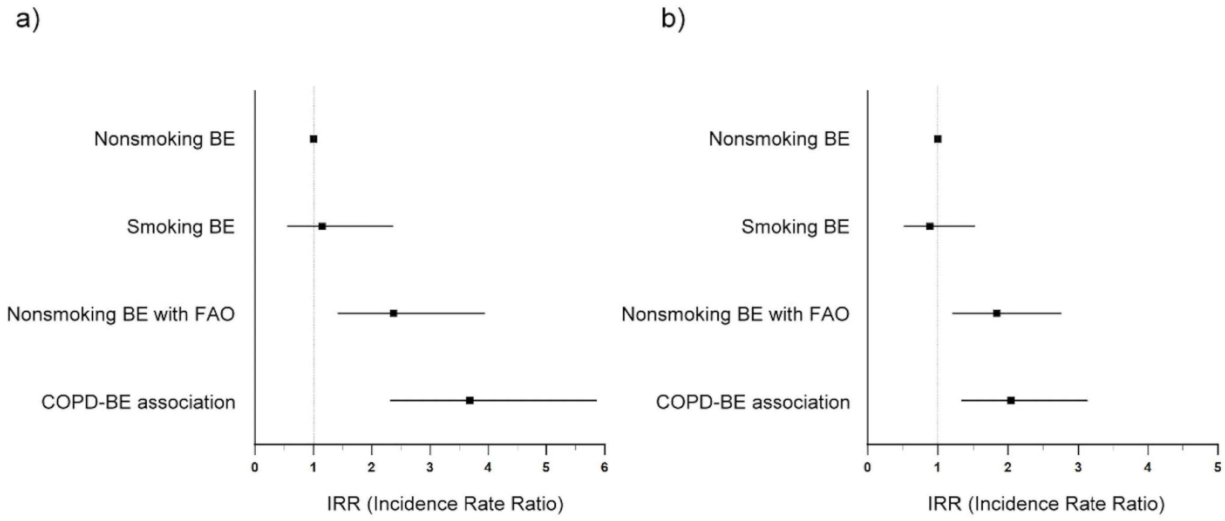

Figure 44. Incidence rate ratios (IRR) for the risk of exacerbation among four subgroups in (a) the prospective cohort and (b) the retrospective cohort of bronchiectasis. Nonsmoking BE was used as the reference group (IRR = 1.0). The IRR values for other subgroups compared to Nonsmoking BE are as follows: Prospective cohort (a): Smoking BE: IRR = 1.14 (95% Confidence Interval [CI]: 0.55-2.37, p=0.709), Nonsmoking BE with FAO: IRR = 2.37 (95% CI: 1.42–3.95, p=0.001), COPD-BE association: IRR = 3.68 (95% CI: 2.32–5.86, p<0.001). Retrospective cohort (b): Smoking BE: IRR = 0.88 (95% CI: 0.51–1.53, p=0.667), Nonsmoking BE with FAO: IRR = 1.83 (95% CI: 1.21–2.76, p=0.004). COPD-BE association: IRR = 2.04 (95% CI: 1.33–3.31, p=0.004).

3.7.5 Clinical outcomes of COPD-BE association and other groups in the retrospective cohort

In the retrospective cohort, **Table 13** shows that both the COPD-BE association and nonsmoking BE with FAO subgroups had similarly higher rates of subsequent exacerbations compared to those with BE without FAO. Consistent with the findings from the prospective cohort, these groups also had a higher risk of subsequent exacerbations

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compared to the nonsmoking BE group, with an IRR of 2.04 (95% CI: 1.33–3.31) for the COPD-BE association group and an IRR of 1.83 (95% CI: 1.21–2.76) for the nonsmoking BE with FAO group (**Figure 44B)**.

Table 14. Effect estimates for clinical outcomes for bronchiectasis patients within the study cohorts stratified by ROSE criteria

Clinical outcomes	N		Exacerbations (OR)	Hospitalization (OR)	Mortality (OR)
Prospective cohort	**147**				
Nonsmoking BE	62	Unadjusted	1.00 (reference)	1.00 (reference)	1.00 (reference)
		Adjusted[#]	1.00 (reference)	1.00 (reference)	1.00 (reference)
Smoking BE	20	Unadjusted	1.25(0.38-4.09)	3.11(0.83-11.60)	6.77(0.58-79.12)
		Adjusted[#]	1.56(0.34-7.08)	3.73(0.63-22.01)	8.41(0.11-633.2)
Nonsmoking BE with FAO	32	Unadjusted	3.32(1.31-8.38)*	2.15(0.63-7.32)	1.96(0.11-32.53)
		Adjusted[#]	3.15(1.02-9.67)*	0.90(0.21-3.81)	0.365(0.12-10.92)
COPD-BE association	33	Unadjusted	4.00(1.60-10.01)*	6.87(2.31-20.42)*	13.5(1.55-118.12)*
		Adjusted[#]	4.06(1.03-15.92)*	6.42(1.16-35.48)*	67.7(0.57-7923.06)
Retrospective cohort	**574**				
Nonsmoking BE	279	Unadjusted	1.00 (reference)	1.00 (reference)	1.00 (reference)
		Adjusted[#]	1.00 (reference)	100 (reference)	1.00 (reference)
Smoking BE	99	Unadjusted	0.81(0.40-1.67)	0.85(0.45-1.61)	8.08(2.10-31.13) *
		Adjusted[#]	0.81(0.36-1.84)	0.87(0.42-1.79)	2.87(0.61-13.44)
Nonsmoking BE with FAO	110	Unadjusted	1.72(0.97-3.07)	1.56(0.91-2.67)	1.70(0.28-10.33)
		Adjusted[#]	1.26(0.66-2.38)	1.13(0.63-2.05)	0.39(0.47-3.35)
COPD-BE association	86	Unadjusted	2.24(1.24-4.07)*	2.32(1.34-4.01)*	10.75(2.84-40.69)*
		Adjusted[#]	2.07(1.00-4.27)*	2.11(1.08-4.11)*	3.39(0.74-16.28)

[#]Adjust for Age, Sex, Diabetes Mellitus, Cardiovascular disease, reflux disease, Old tuberculosis infection, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, modified Reiff score, Inhaled corticosteroids, Macrolides use, *p<0.05.

Abbreviations: BE = Bronchiectasis; COPD = Chronic obstructive pulmonary disease; FAO=Fixed airflow obstruction.

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This indicates that patients in the COPD-BE association and nonsmoking BE with FAO groups exhibited a higher risk of exacerbations compared to those without FAO. However, after adjusting for confounding factors during follow-up, we found that only the COPD-BE association group exhibited a significantly higher risk of exacerbation (aOR: 2.07, 95% CI: 1.00–4.27) (**Table 14**).

3.7.6 The bronchiectasis patients with a clinical diagnosis of COPD had worse outcomes

Table 15 highlights the significant impact of COPD diagnosis in bronchiectasis cohorts. In the prospective cohort, 38.1% (56 of 147) of bronchiectasis patients were co-diagnosed with COPD, while in the retrospective cohort, this figure was 54.5% (313 of 574). Patients with COPD co-diagnosis had poorer lung function, with a higher percentage of post-bronchodilator $FEV_1/FVC < 0.7$ (73.2% prospective, 52.7% retrospective) and greater smoking exposure (53.6% prospective, 42.2% retrospective). Meeting the ROSE criteria was more common in patients with a diagnosis of COPD (39.3% prospective, 24.3% retrospective).

Regarding medication use, patients with COPD co-diagnosis in the prospective cohort more frequently used inhalation therapies, with similar trends observed in the retrospective cohort. Notably, ICS was also more commonly used in those with co-diagnosis of COPD (12.5% vs. 5.5% prospective, 16.9% vs. 7.7% retrospective). Clinical

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outcomes were worse in patients with COPD co-diagnosis, with higher rates of exacerbations (44.6% vs. 27.5% prospective, 20.1% vs. 11.5% retrospective) and hospitalizations (30.4% vs. 15.4% prospective, 24.3% vs. 16.1% retrospective) compared to those without a diagnosis of COPD.

Table 15: COPD diagnosis and ROSE criteria in prospective and retrospective cohorts of bronchiectasis

	Prospective cohort (N=147)			Retrospective cohort(N=574)		
	No COPD diagnosis	**COPD diagnosis**	**P value**	**No COPD diagnosis**	**COPD diagnosis**	**P value**
Number, n(%)	**91(61.9)**	**56 (38.1)**		**261(45.4)**	**313(54.5)**	
Post BD $FEV_1/FVC < 0.7$, n (%)	24(26.4)	41(73.2)	<0.001*	31(11.9)	165(52.7)	<0.001*
Post BD FEV_1/FVC, median (IQR)	75.8(68.8-80.2)	65.3(59.4-70.2)	<0.001*	79.7(74.5-86.2)	69.2(60.6-77.1)	<0.001*
Smoking exposure $\geq$ 10 pack-years, n(%)	23(25.3)	30(53.6)	0.001*	53(20.3)	132(42.2)	<0.001*
Modified Reiff score, median (IQR)	4.0(2.0-6.0)	4.0(3.0-5.75)	0.495	5.0(3.0-7.0)	5.0(3.0-8.0)	0.950
Meet ROSE criteria, n(%)	11(12.1)	22(39.3)	<0.001*	10(3.8)	76(24.3)	<0.001*
Inhalation therapies, n(%)						
Monotherapy (LAMA or LABA)	6(6.6)	11(19.6)	0.016*	22(8.4)	65(20.8)	<0.001*
LAMA+LABA	24(26.4)	36(64.3)	<0.001*	40(15.3)	123(39.3)	<0.001*
Triple therapy	5(5.5)	7(12.5)	0.213	5(1.9)	38(12.1)	<0.001*
Inhaled corticosteroid (ICS)	5(5.5)	7(12.5)	0.213	20(7.7)	53(16.9)	0.001*
Macrolides	15(16.5)	10(17.9)	0.830	26(10.0)	37(11.8)	0.283
Clinical outcomes, n (%)						

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Exacerbation	25(27.5)	25(44.6)	0.033*	30(11.5)	63(20.1)	0.005*
Hospitalization	14(15.4)	17(30.4)	0.031*	42(16.1)	76(24.3)	0.016*
Mortality	5(5.5)	5(8.9)	0.506	6(2.3)	16(5.1)	0.080

Data are presented as No. (%) or median (interquartile range), unless otherwise indicated

For each row, data are either % with p-values from t test or Fisher's exact tests between the two groups, median

(IQR) with p values from Mann-Whitney tests; *p <0.05.

Abbreviations: BD=bronchodilator; COPD = Chronic obstructive pulmonary disease; FEV_1 = forced expiratory volume

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Chapter 4. Discussion

Our study represents a pioneering effort to analyze lung microbiota and airway inflammation in bronchiectasis patients with FAO from an East Asian population, comparing these patients with those having COPD and bronchiectasis without FAO using BAL samples. We discovered that the lung microbiota in patients with BE-FAO closely resembled that of patients with bronchiectasis, with both groups exhibiting reduced microbial diversity and a predominance of *Proteobacteria* compared to COPD patients alone. Bronchiectasis patients with FAO demonstrated greater neutrophilic airway inflammation and a higher risk of exacerbations than those with COPD or bronchiectasis alone. Importantly, we identified a positive correlation between *Pseudomonas aeruginosa* colonization and increased airway neutrophilic inflammation, along with a higher BSI score, potentially indicating a predictor for future exacerbations in the BE-FAO group. Furthermore, this is the first study to investigate two distinct entities within the BE-FAO group based on the ROSE (Radiology, Obstruction, Symptoms, and Exposure) criteria, revealing two unique endotypes characterized by their clinical characteristics, inflammatory patterns, and microbiome compositions.

We further applied the ROSE criteria to identify patients categorized as “BE-FAO ROSE (+)”, commonly referred to as the “COPD-BE association”, across both prospective and retrospective multicenter cohorts. These patients demonstrate a higher

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risk of future exacerbations and hospitalizations compared to other bronchiectasis groups. These findings highlight the need for tailored management strategies. Future research should prioritize long-term outcomes to enhance our understanding of bronchiectasis progression, particularly in subgroups with frequent exacerbations, regardless of smoking status.

Bronchiectasis and COPD often coexist, leading to the terms “COPD-bronchiectasis association” [76] or “Bronchiectasis-COPD overlap” [71,74]. This overlap is associated with increased airway inflammation, more clinical symptoms, greater disease severity, and a worse prognosis than either disease alone [71,72,74, 82,116]; these findings are consistent with our study. Differing from previous studies [75,78] our research extended beyond just bronchiectasis patients meeting the ROSE criteria, commonly referred to as the “COPD-bronchiectasis association” [76]. We also included non-smoking advanced bronchiectasis patients in the BE-FAO group, which could be classified as “BE-FAO ROSE (-)”. Our results indicated that patients with BE-FAO, whether ROSE (+) or ROSE (-), and those with bronchiectasis alone, had comparable lung microbiomes. These findings are consistent with those of Huang et al. [75], who also employed the ROSE criteria. However, in contrast to the UK cohort [75], we observed that alpha diversity in BE-FAO ROSE (+) was similar to COPD, with less distinct beta diversity. The differences between the cohorts could be attributed to several factors: (1) The majority of

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our COPD and BE-FAO ROSE (+) cohort are males (> 95%), more likely to have smoking habits [80,117] than the non-Asian cohort [75]. (2) Geographic variations in lung microbiome influenced by different COPD [80,81,117] and bronchiectasis [82,83] risk factors and etiologies. (3) Environmental exposure, such as air or indoor pollution, along with geographic differences and varying dietary exposures, impacts the airway microbiome [79,81,82,83]. (4) Ethnic-based differences in microbiome and host immunity interactions could also be a contributing factor [79,82,83].

Moreover, although the ROSE criteria effectively stratify patient groups, their primary reliance on smoking history may oversimplify the complexities of disease dynamics. These criteria do not account for other significant factors affecting disease development, such as genetic or environmental influences (e.g., exposure to indoor pollution) or pre-existing comorbidities. Thus, broader criteria should be established. Further research involving validation cohorts from diverse geographic regions is essential to expand these findings and provide a more comprehensive understanding of the multifactorial influences on diseases.

Furthermore, patients with the two disease entities of BE-FAO exhibited similar microbial diversity, with overlapping lung microbiota communities. Nevertheless, subtle differences emerged at the phyla and species levels. For instance, *Candidatus Absconditabacteria* was more common in BE-FAO ROSE (+), while species such as

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Pseudoleptotrichia goodfellowii and *Streptococcus mutans* were more prevalent in BE-FAO ROSE (-). Moreover, compared to patients with BE-FAO ROSE (+) or the "COPD-bronchiectasis association", those with BE-FAO ROSE (-) were predominantly female and tended to have an idiopathic etiology, exhibited greater airway neutrophilic inflammatory cytokines, and had a lower emphysema score. However, the radiological severity of bronchiectasis, the degree of lung function obstruction, and exacerbation outcomes were similar between both entities. Given the variations in clinical features, etiologies, inflammatory profiles, and lung microbiome between these two entities of bronchiectasis with FAO, we hypothesize that they might represent distinct biological and microbiological endotypes.

Our analysis revealed a positive association between *Pseudomonas aeruginosa* colonization and neutrophilic inflammation, as well as higher severity of bronchiectasis in patients with FAO. This indicates a "*Proteobacteria*-neutrophilic endotype" in the COPD–bronchiectasis association [75], potentially contributing to a higher risk of future exacerbations in BE-FAO and serving as a biomarker for poorer prognosis [74,77,118]. In contrast, the lung microbiome of patients with COPD exhibited greater diversity with a dominance of the *Firmicutes* phylum and commensal taxa, differing from those in BE and BE-FAO. This diversity suggests "diverse endotypes" in the COPD–bronchiectasis association [75], potentially associated with a lower risk of exacerbation compared to

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bronchiectasis with FAO. Prior research links high blood eosinophils with a *Firmicutes*-dominated microbiome [64,66] supporting the effectiveness of inhaled corticosteroids (ICS) in COPD [66,119]. The impact of eosinophils on the bronchiectasis microbiota is an area of growing interest [120, 121], with potential ICS benefits for specific bronchiectasis subgroups [121, 122]. Our East Asian cohort showed no clear correlation between blood eosinophils and specific lung microbiota in terms of clinical outcomes. Nevertheless, the directionality and causality of the relationship between airway inflammation and lung microbiome remain unclear. Longitudinal studies are crucial to ascertain whether changes in microbiota precede or follow changes in inflammation and to explore ongoing changes in the lung microbiome and inflammatory markers.

Another novel finding from our study is the positive association of two anaerobic oral taxa, *Treponema socranskii* and *Dialister invisus*, commonly detected in periodontitis [123,124] with airway neutrophilic inflammation in the exacerbation subgroup of the BE-FAO group. This suggests that microaspiration-derived microbiota contribute to lung inflammation [125] and are associated with defective mucosal immunity in patients with chronic lung diseases [126,127,128]. Nevertheless, the specific role of the oral microbiome in patients with bronchiectasis and FAO, particularly its interaction with mucociliary clearance dysfunction, remains unexplored. Further studies

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are essential to investigate these relationships and their potential implications in the pathogenesis of these conditions.

To further investigate the clinical outcomes of bronchiectasis and frequent exacerbations (FAO) in relation to the ROSE criteria, we applied these criteria to an East Asian cohort to identify patients with a COPD-BE association. Analyzing data from both prospective (n=147) and retrospective (n=574) cohorts in Taiwan, we found that 16.5% of participants, primarily older males, were classified as having a COPD-BE association.

Our findings also show that patients with COPD-BE association were significantly older and predominantly male compared to the nonsmoking BE group. This observation suggests that long-standing disease and male gender —particularly because males are more likely to smoke and develop COPD in Taiwan [80]— may be risk factors for developing COPD-BE association. Consistent with previous reports [75,129], patients with COPD-BE association had more dyspnea, poorer lung function, and a higher prevalence of COPD diagnoses compared to those without airflow obstruction in both cohorts. Notably, in our prospective cohort, we demonstrated that patients with COPD-BE association had higher neutrophilic inflammatory markers, suggesting a more pronounced inflammatory response [68,69,75]. Moreover, blood eosinophil levels were significantly higher in smoking groups compared to those without smoking exposure, as described in a previous report [130].

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We emphasized the comparison between patients with COPD-BE association and those with nonsmoking BE with FAO. In the prospective cohort, both groups showed similar clinical symptoms, lung function, bronchiectasis severity, and comorbidities. However, in the retrospective cohort, COPD-BE patients were slightly older, had higher BMI, lower FEV1/FVC ratio, and less radiological severity compared to nonsmoking BE with FAO patients. This discrepancy may result from the potential impact of different sample collection methods, leading to varying disease severity between the two cohorts. Furthermore, we found that approximately 40% of patients with bronchiectasis without airflow obstruction used long-acting bronchodilators in both cohorts. This overuse of long-acting bronchodilators has also been observed in other real-world data [131,132,133], likely due to factors such as the high prevalence of dyspnea in bronchiectasis, even in the absence of airway obstruction [95]. Dyspnea severity in bronchiectasis often correlates poorly with FEV_1 decline and disease severity [134]. Some physicians may also consider that small airway dysfunction and pulmonary hyperinflation play significant roles in the pathophysiology of dyspnea in these patients, leading to the use of bronchodilators even in the absence of obstructive airflow limitation [135,136], which may also contribute to the overdiagnosis of COPD.

Microbiologically, the prospective cohort had a higher yield rate than the retrospective cohort, likely due to different collection methods: BAL samples versus

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sputum samples. Slight differences emerged between the groups: nonsmoking BE with FAO had a higher prevalence of *Pseudomonas aeruginosa*, while the COPD-BE group had more *Klebsiella pneumoniae*. Both groups had a significantly higher risk of exacerbation compared to those with nonsmoking BE, consistent with previous reports [75,95,129]. This highlights the importance of these bacteria in exacerbation outcomes [68,75, 82,97,129,137].

Comparing the clinical outcomes of COPD-BE association and nonsmoking BE with FAO, both groups showed similar clinical exacerbation risks in both cohorts. However, after adjusting for confounding factors, only individuals with COPD-BE association—not nonsmoking BE with FAO—had markedly increased risks of exacerbation and hospitalization compared to nonsmoking BE in both cohorts. This finding underscores the potential for these groups to represent distinct clinical entities with significant differences in disease progression and long-term outcomes, highlighting the need for nuanced risk stratification, particularly for bronchiectasis patients with a history of smoking and obstructive lung function.

Our study, integrating data from two cohorts, revealed that over half (51.1%, 369 of 741) of bronchiectasis patients had a co-diagnosis of COPD, significantly higher than previously reported [129]. This discrepancy may be due to selection bias, as the retrospective cohort included only patients with documented lung function, and clinicians

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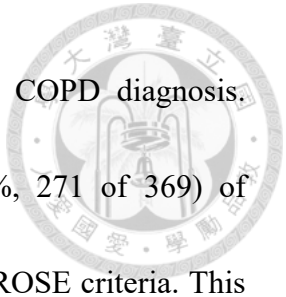

are more likely to order lung function tests when considering a COPD diagnosis. Additionally, our data indicated that a substantial portion (73.4%, 271 of 369) of bronchiectasis patients with a COPD diagnosis did not meet all the ROSE criteria. This overdiagnosis of COPD may partly be due to the large proportion (29.2%, 108 of 369) of non-smoking BE patients with FAO who often had a co-diagnosis of COPD. Since we did not have detailed exposure data, such as biomass or air pollution exposure in Taiwan, it is challenging to clearly determine whether these COPD diagnoses are attributed to nonsmoking-related exposures (nonsmoking COPD), which could also include COPD-BE association with biomass exposure [76] or are a result of long-standing disease and advanced bronchiectasis leading to FAO. Further research is needed to clarify these findings. Conversely, we also identified patients who met the ROSE criteria but did not have a COPD diagnosis. These patients were less common (5.9%, 21 of 352) among those without a COPD diagnosis, consistent with previous reports [129]. Our study also confirmed that clinical outcomes were notably worse in patients with a co-diagnosis of COPD [129], with higher rates of exacerbations and hospitalizations in both cohorts. These findings highlight the need for careful management and targeted therapeutic strategies for bronchiectasis patients with a co-diagnosis of COPD, given their poorer prognosis and increased healthcare utilization.

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The strength of this study lies in its use of the comprehensive, validated ROSE criteria in an East Asian population, applied within both prospective and large retrospective bronchiectasis cohorts, excluding patients with clinical asthma and ABPA. In the prospective study, we utilized BAL samples at a single center, necessitating validation across multiple cohorts. The inclusion of a large retrospective cohort (n=574) from 16 hospitals across Taiwan enhances the generalizability of our results, allowing for a more representative analysis of the East Asian population. Differences in sample collection methods between the prospective (BAL) and retrospective (sputum) cohorts may account for variations in observed disease severity, with sputum samples possibly reflecting more severe cases. This study provides additional insights into the COPD-BE association in an East Asian population, highlighting potential differences in disease presentation and outcomes when compared to non-Asian cohorts. Our findings underscore the importance of considering genetic, environmental, and cultural factors in managing these conditions across diverse populations. Future research should focus on long-term follow-up to better understand bronchiectasis progression in subgroups with FAO, regardless of smoking status, as these may represent distinct disease entities influenced by varying demographics or microbiological profiles. Consistent studies on microbiological factors and airway inflammation across cohorts are also crucial for deeper insights into disease mechanisms. Clinically, these findings highlight the need for

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tailored management strategies, considering the influence of smoking and comorbid conditions on outcomes.

Study Limitations

Our studies have several limitations that warrant acknowledgment.

First, as a prospective cross-sectional observational cohort study in lung microbiome research, it identifies associations rather than causality. This emphasizes the need for longitudinal studies to clarify the causal links between the lung microbiome, airway inflammation, and clinical outcomes. Additionally, our reliance on 16S rRNA gene sequencing limits our ability to identify specific bacterial species or strains and does not provide functional information about the lung microbiome; whole genome sequencing (WGS) is recommended for a more comprehensive understanding of the mechanistic pathways involved.

Second, we enrolled only clinically stable patients to ensure safety during bronchoalveolar lavage collection and to minimize the effects of recent antibiotic exposure. Consequently, our findings may not apply to patients experiencing exacerbations or those with more severe disease. Future research should include a broader patient population, encompassing individuals with exacerbations, those undergoing antibiotic treatment, and healthy controls, to comprehensively assess microbiome dynamics across different disease states.

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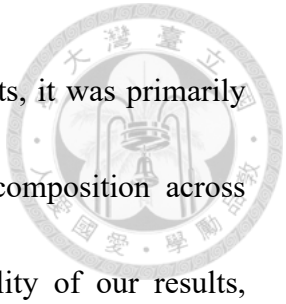

Third, while our lung microbiome study offers valuable insights, it was primarily conducted in East Asian populations. Differences in microbiota composition across geographic regions and ethnic groups may limit the generalizability of our results, necessitating larger multicenter trials to substantiate and broaden our findings. Moreover, the sample size within each group may not adequately represent their respective populations, potentially limiting statistical power, especially in detecting minor effects or rare microbial species. Future studies with larger sample sizes are warranted to clarify these results.

Fourth, in our two validated cohorts, the prospective cohort included fewer participants, while over 50% of patients in the retrospective cohort lacked detailed lung function and/or smoking history, resulting in their exclusion. The one-year follow-up for the retrospective cohort may not sufficiently capture long-term outcomes, potentially introducing biases. Moreover, while the application of the ROSE criteria offered robust stratification based on clinically relevant parameters, it may have introduced bias, particularly in identifying a higher prevalence of obstruction in the COPD-BE association and nonsmoking BE with FAO groups. Nevertheless, this stratification enabled a more detailed analysis of subgroup differences beyond smoking status and lung function.

Lastly, differences in microbiological data collection methods—BAL samples in the prospective cohort versus sputum samples in the retrospective cohort—may have

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introduced discrepancies in comparison. Additionally, we did not compare the "pure COPD" cohort, limiting our ability to highlight differences between COPD and COPD-BE associations as presented in previous reports [75,129,138]. The lack of comprehensive exposure data beyond cigarette smoking potentially excluded some non-smoking bronchiectasis cases from our "ROSE" group. As a retrospective real-life registry, microbiological sampling was only performed when clinically indicated, resulting in missing data points and challenges in accurately distinguishing between chronic airway diseases such as COPD, allergic bronchopulmonary aspergillosis (ABPA), and pulmonary fibrosis with tractional bronchiectasis.

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Chapter 5 Conclusion and Future Prospects

In the East Asian cohort, bronchiectasis with fixed airflow obstruction (BE-FAO) is markedly distinct and clinically more severe compared to COPD or bronchiectasis alone, exhibiting increased neutrophilic inflammation and a higher risk of exacerbations. Both bronchiectasis with and without FAO, characterized by reduced microbial diversity and dominant *Proteobacteria*, share similar microbiome compositions that are distinct from COPD alone. Utilizing the ROSE criteria, our study identified two distinct endotypes within the BE-FAO group, differentiated by their clinical features, inflammatory patterns, and microbiome attributes. Notably, a significant correlation was observed between *Pseudomonas aeruginosa* colonization and heightened airway neutrophilic inflammation in BE-FAO patients, along with an increased bronchiectasis severity. This relationship may serve as an indicator of potential future exacerbations in the BE-FAO group.

Our study further supports the effective use of the ROSE criteria to identify patients with COPD-bronchiectasis (COPD-BE) association in East Asian populations, who exhibit higher risks of future exacerbations and hospitalizations compared to other bronchiectasis groups. These findings underscore the necessity for tailored management strategies. Future research should prioritize long-term outcomes to better understand

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bronchiectasis progression, particularly in subgroups with FAO, regardless of smoking status.

Looking ahead, we aim to gain deeper insights into the impacts of the identified endotypes on disease progression and treatment outcomes through shotgun metagenomics gene sequencing. This approach will explore the underlying mechanisms driving these differences. While our current study does not address therapeutic applications directly, we plan to expand our research to include treatment modalities such as azithromycin [139] and inhaled antibiotics (e.g., colistin) and examine their interactions with the lung microbiome and gut microbiome—often referred to as the gut-lung axis. Additionally, we will use interactome analysis to evaluate the fungal mycobiome and its interactions with the bacteriome [140]. Through these efforts, we hope to optimize care for patients with bronchiectasis and COPD.

In conclusion, our findings pave the way for a more nuanced approach to managing bronchiectasis and COPD, emphasizing the importance of individual patient characteristics and microbiome dynamics in future therapeutic strategies. As we advance our research, our ultimate goal remains to translate these insights into effective interventions that improve the quality of life and clinical outcomes for affected individuals.

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Appendix. Publications (2011-2024)

1. **Chen YF**, Chen CY, Hsu CL, Yu CJ. Malpositioning of the chest tube across the anterior mediastinum is risky in chronic obstructive pulmonary disease patients with pneumothorax. *Interact Cardiovasc Thorac Surg*. 2011; **13** :109-11.
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Positive Lung Adenocarcinoma Compared with in Patients Harboring Other Driver Mutations in East Asian Populations. *J Thorac Oncol.* 2016; **11** : 1140-52.

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