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PhD thesis on

Airway microbiome and host inflammatory response in bronchiectasis

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Abstract

Airway microbiome and host inflammatory response in bronchiectasis

Non-cystic fibrosis bronchiectasis (hereby referred to as bronchiectasis) is a chronic respiratory disease characterized by irreversible dilation of bronchi in the context of chronic syndrome mainly represented by daily cough, sputum production and frequent pulmonary exacerbations. Bronchiectasis pathophysiology is recognised in the development of a vicious circle of infection, inflammation, mucous clearance and pulmonary damage. Although microbial community and inflammation have a very important role in respiratory diseases, few is known in bronchiectasis. The aim of this PhD project was to evaluate sputum microbiome and its interaction with the local host inflammatory response in adults with bronchiectasis during their stable state. Several studies were conducted to explore this research question.

In the second chapter, we report the methodological selection of techniques for both microbiome and active neutrophil elastase (aNE) analysis in sputum.

The third chapter is divided into three different sections. In the first, we identified two groups of adult bronchiectasis patients with different microbiome diversity levels. The low microbiome diversity group was found to be enriched in *Pseudomonas*, *Staphylococcus* and *Streptococcus*. Multivariate analysis identified FEV_1%predict.<50, radiology and primary ciliary dyskinesia (PCD) to be independently associated with low microbiome diversity.

The following section represents a preliminary study focused on bronchiectasis aetiologies, more specifically PCD and immunodeficiency. The study concerning PCD did not identify a clear association between aetiology, microbiome and inflammation comparing a group of matched PCD with idiopathic or post infective bronchiectasis, although, a trend could be observed. Subsequently, immunodeficiency was comparedto idiopathic bronchiectasis. These two groupsidentified, primary immunodeficiency and idiopathic differed in terms of alpha diversity and quantification of *H. influenzae* which was higher in the immunodeficiency group.

Chapter four focuses on neutrophilic inflammation looking at the association of aNE in bronchiectasis with microbiome analysis. We found evidences of low microbiome diversity and *P. aeruginosa* identification in patients with high levels of aNE.

The following study focused on active Cathepsin G (Cat-G) which is a putative biomarker for bronchiectasis. Cat-G was associated with disease severity, radiological severity, quality of life and chronic infection. It was also associated with low microbiome diversity and *P. aeruginosa* molecular detection. Finally, Cat-G and aNE resulted to be similar in predicting bronchiectasis severity (bronchiectasis severity index -BSI), severe exacerbation and chronic infection in bronchiectasis patients during stable state.

Finally, in chapter five, we analyse microbiome and inflammation among adult bronchiectasis patients with chronic *P. aeruginosa* infection. Association network analysis identified differences in terms of number of interactions of *Pseudomonas* with other genera and interactions between cytokines and microbial effectors in patients with different exacerbations/year.

We hope that this thesis may be a step forward in better understanding the role of both microbiome and inflammation in bronchiectasis that may lead to unravelling of endotypes and the identification of therapeutic targets. This will finally result in advances in a precise medicine approach for bronchiectasis patients.

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List of abbreviations

AAT	Alpha-1-Antitrypsin
ABPA	Allergic Bronchopulmonary Aspergillosis
aNE	Active Neutrophil Elastase
ASV	Amplicon Sequence Variants
AUC	Area Under the Curve
BACI	Bronchiectasis Aetiology Comorbidity Index
BAL	Bronchoalveolar Lavage
BAS	Bronchial Aspirate
BE	Bronchiectasis
BMI	Body Mass Index
BSI	Bronchiectasis Severity Index
Cat-G	Active Cathepsin G
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CFTR-RD	CFTR-Related Disorders
COPD	Chronic Obstructive Pulmonary Disease
CS	Enzymatic Digestion Assays Using Chromogenic Substrate
CT scan	Computed Tomography Scan
CVID	Common Variable Immunodeficiency
DPPI	Dipeptidil Peptidasi I
DTT	Dithiothreitol
ENOS	Effective Number Of Species
ERS	European Respiratory Society
FEV_1	Forced Expiratory Volume In The 1st Second
FS	Enzymatic Digestion Assays Using Fluorogenic Substrate
GBLMs	Generalized Boosted Linear Models
GERD	Gastro-Oesophageal Reflux Disease
HIV	Human Immunodeficiency Virus
ICC	Intraclass Correlation
ICS	Inhaled Corticosteroids
Ig	Immunoglobulin
IL	Interleukin
IQR	Interquartile Ranges
LABA	Long-Acting B Adrenoceptor Agonists
LAMA	Long-Acting Muscarinic Antagonists
MIP2	Macrophage Inflammatory Protein 2
mMRC Dyspnoea Scale	Modified Medical Research Council Dyspnoea Scale
MRSA	Methicillin-Resistant Staphylococcus Aureus
MSSA	Methicillin-Sensitive S. Aureus
NE	Neutrophil Elastase
NGS	Next-Generation Sequencing
NMDS	Nonmetric Multidimensional Scale
NSP	Serine Proteases

NTM	Non-Tuberculous Mycobacteria
OTUs	Operational Taxonomic Units
PBS	Phosphate-Buffered Saline
PCD	Primary Ciliary Dyskinesia
PCoA	Principal Coordinate Analysis
PPI	Proton-Pump Inhibitor
QIIME	Quantitative Insights Into Microbial Ecology
QoL-B	Quality Of Life In Bronchiectasis Questionnaire
ROC	Receiver Operating Characteristic
ROS	Reactive Oxygen Species
rRNA	Ribosomal RNA
TNFα	Tumour Necrosis Factor α

Chapter 1

1 General introduction

1.1 Bronchiectasis

Bronchiectasis is an abnormal and permanent dilatation of the bronchi associated with a chronic respiratory syndrome. Daily symptoms are mostly represented by chronic cough and the production of large volumes of dense, viscous and purulent sputum, in a context of chronic infection, inflammation and frequent exacerbations [1]. Bronchiectasis is a very heterogeneous disease from different points of view. This heterogeneity permeates all the disease components, including symptoms, aetiology, airway infection and host biological response. Bronchiectasis are a distinctive trait of cystic fibrosis (CF). CF and non-CF bronchiectasis are very different in terms of aetiology, treatment and prognosis. Objective of this thesis are non-CF bronchiectasis (referred to from now on only as bronchiectasis).

1.2 Epidemiology

Bronchiectasis has been considered for many years as an orphan disease while its prevalence increased over the last two decades [2].

Different studies showed a prevalence ranging between 32 and 67 cases per 100 000 (Catalonia-Spain, 2012 and Germany, 2005-2011) in Europe. In the UK in 2013 prevalence was 566.1 per 100000 for women and 485.5 per 100000 for men, ranging around 1% in both men and women in elderly [3, 4]. Retrospective studies in the USA reported a prevalence of 139/100 000 in the general population, with an increase in women (180/100 000) and in elderly (age ≥75 812/100 000) between 2009 and 2013 [5]. Prevalence in China between 2002 and 2004 was reported to be 1.2% above the age of 40 [6]. Finally, a recent experience reported a prevalence in Italy of 163 per 100,000 population. The prevalence increased with age and was slightly higher in women than in men [7]. Other experiences reported an increased prevalence and a higher disease severity in women and with an increasing age. These differences may be potentially associated with both microbiome and immune system changes for age and immune system gender-associated differences [8].

1.3 Aetiology

The aetiology of bronchiectasis is very heterogeneous. The investigation of different aetiologies may lead to the identification of potentially treatable causes and it is crucial for a personalised treatment. A European study performed in 2015 reported a 13% of patients with a treatable cause of the disease [9]. Nevertheless, in many cases the identification of an aetiology cannot be reached despite the diagnostic tests suggested by the international guidelines put in place, and, in this case, bronchiectasis is reported as "idiopathic". The other most frequently reported causes of bronchiectasis in Europe include 20% post-infectious, 15% chronic obstructive pulmonary disease (COPD), 10% connective tissue diseases, 5.8% immunodeficiency and 3.3% asthma [9]. In about 40% of the cases, bronchiectasis isidiopathic [10]. Furthermore, aetiology differs region by region; in India, post-infectious bronchiectasis reached 41% and 16% of patients was reported as post-tuberculosis in China. Moreover, idiopathic bronchiectasis was reported up to 66% in China [11]. In the USA, the multi-ethnic population allowed the scientific community to identify aetiological differences among bronchiectasis patients. The most common aetiology in the USA was reported to be immune related [12]. Data reported in literature confirm a high influence of the environment/climate in the pathogenesis of bronchiectasis [12].

1.3.1 Immunodeficiency

Immunodeficiency is one of the causes of bronchiectasis. Immunodeficiency may be divided in primary and secondary immunodeficiency.

1.3.1.1 Primary immunodeficiency

Defects at immunological level may be a cause of bronchiectasis. Chronic syndromes can be included in primary immunodeficiency and common variable immunodeficiency (CVID) is one of these. CVID is characterized by reduced plasma levels of IgG and IgA or IgM with a normal lymphocyte B count. CVID may be a cause of frequent pulmonary infections leading to the development of pulmonary diseases (mostly bronchiectasis), autoimmune diseases and increased risk of cancer development. [13]. Other disorders, like isolated deficit of IgA, IgG subclasses or IgM, that are usually asymptomatic in the general population may be associated to an increased risk of bronchiectasis development [14, 15]. Defects at antibodies levels may represent impairment of bacterial opsonisation and causes may be found at different levels of lymphocyte B maturation [16]. Immunoglobulin deficit may be treated with specific supplementation [17]. In other cases, mononucleated phagocytes are involved in the immunodeficiency. These antigen-presenting cells are able to recruit cellular immune response and diseases involving these cells

increase the susceptibility of patients to intracellular pathogens infections as non-tuberculous mycobacteria (NTM) [18].

1.3.1.2 Secondary immunodeficiency

Secondary immunodeficiency may be involved in bronchiectasis development. HIV infection is included in this category. Other immunosuppressive conditions may be consequences of treatment of autoimmune or cancer therapies. Specifically, association among Rituximab administration and respiratory infections and bronchiectasis was described in literature. [19]

1.3.2 Post infective

Post infective aetiology is reported if the patient has an onset of symptoms after a severe respiratory infection such as pneumonia or tuberculosis, regardless the latency between the event and the onset of the diseases. Furthermore, the identification of this cause of bronchiectasis is difficult due to the lack of scientific agreement in the definition of this aetiology [20]. Post-tuberculous bronchiectasis are the most represented aetiology in Asia, due to high incidence of tuberculosis. Post infective bronchiectasis is frequently caused by sub optimally treated either viral or bacterial pneumonia or pneumonia relapse in children [12].

1.3.3 NTM infection

Non-tuberculous mycobacteria (NTM) are intracellular pathogens frequently colonizing bronchiectasis lungs. NTM infection may lead to the development of NTM pulmonary disease that could cause broncodilation and bronchiectasis. Moreover, bronchiectasis patients seem to have a higher susceptibility to the development of this infection. When NTM infection is diagnosed, it is difficult to understand if the microorganism is the causing factor *versus*a consequence of the diseases. Treatment of this infection is pharmacological. The use of a long-term cocktail of antibiotics is needed to fully eradicate this pathogen [21–24].

1.3.4 Asthma and COPD related bronchiectasis

The association between bronchiectasis and other chronic respiratory diseases such as asthma and COPD is frequent. Poor control of asthma or the continuous immunosuppression due to exposition to inhaled corticosteroids may lead to the development of pulmonary infections [25]. COPD-bronchiectasis overlap syndrome is also frequent and characterized by a specific phenotype of higher disease severity and increased mortality compared to COPD patients [26, 27].

1.3.5 Genetic causes

1.3.5.1 CFTR-Related Bronchiectasis

CF is a genetic recessive disease. Two mutations in CFTR gene are needed for the onset of this systemic disease. Borderline situations where the subject is a carrier of the disease with one only mutation or with two mutations not able to fully compromise CFTR functions are at risk of developing CFTR-related diseases, as CFTR-related bronchiectasis [28]. These patients will result in partially functional CFTR with borderline sweat-test values.

1.3.5.2 Primary ciliary dyskinesia (PCD)

PCD is an autosomal recessive disease characterized by a genetic defect in the dyenin protein. This mutation causes a functional deficiency in cilia development and movement. PCD is a systemic syndrome with respiratory implications. Mucociliary clearance is impaired, resulting in a retention of bronchial secretions and the development of frequent respiratory infections. Furthermore, ciliary movement is also very important during development, being responsible of organs rotation in thorax and abdomen. Half of these patients show a peculiar condition of total or partial *situsinversus*. Ciliary impairment may increase fertility problems [29].

1.3.5.3 Alpha-1-antitrypsin (AAT) deficiency

Alpha-1-antitrypsin AAT is an albumin like protein responsible for the inhibition of serine proteases. It is coded by SERPINA1 gene and mutations at this level may produce partially functional proteins [30]. The sign and symptoms first develop in lungs between 20 and 50 years of age. This protein is responsible for the 90% of the inhibition of serine proteases released from neutrophils [30]. The unopposed activity of these proteases may cause pulmonary defects like bronchiectasis. This deficiency may be treated by pharmacological supplementation of AAT protein [30].

1.3.6 Idiopathic bronchiectasis

When a cause of bronchiectasis may not be defined from diagnostic tests, the term idiopathic is used. In order to homogenize aetiologies and decrease idiopathic bronchiectasis numbers the scientific community agreed on the definition of guidelines for diagnostic tests [1]. The 2017 ERS guidelines suggested a bundle of diagnostic test in order to investigate the most common causes of bronchiectasis [1]. Moreover, Araujo and colleagues suggested an algorithm able to standardize aetiologies definition [11].

1.4 Disease severity

The classification of patients based on disease severity is very important in bronchiectasis in both clinical practice and research, from prognosis to treatment evaluation and disease follow up. Moreover, patients' stratification is very important in order to select the most suitable population for clinical trials.

Several disease severity scores are available and used in clinical practice and research and included in this thesis:

- BSI score (Bronchiectasis severity index- 3 risk classes: 0-4 mild, 5-8 moderate, > 8 severe). This score includes age, BMI, FEV_1, chronic infection, mMRC, radiological damage, exacerbations (the year before) and hospitalizations (two years before) [31]
- FACED score (3 risk classes: 0-2 mild, 3-4 moderate, 5-7 severe). This score includes age, FEV_1, chronic infection, mMRC, radiological damage. E-FACED is an extended score adding to FACED 2 extra point for exacerbations (the year before) [32].
- BACI score (3 risk classes: 0 mild, 1-5 moderate, <8 severe). This score includes patients' comorbidities [33].
- Reiff score: radiological severity score, it consists in an evaluation of chest CT scan. It sets a score for bronchiectasis morphology and number of lobes. The score goes from 0 to 18 in a patient with cystic bronchiectasis at 6 lobes [34].

1.5 Symptoms and quality of life

Symptoms associated to bronchiectasis are very heterogeneous and may vary from patient to patients. Patients develop daily cough with daily production of large amounts of sputum, shortness of breath and wheezing, haemoptysis and frequent exacerbations. Symptoms may have a high impact on quality of life and, in some cases, represent a lifelong social *stigma*. Furthermore, the frequency of exacerbations may further increase this psychological aspect inducing anxiety and depression in bronchiectasis patients.

In order to have an objective representation of quality of life, different questionnaires were developed. One of the most commonly evaluated in bronchiectasis, which will be used through this thesis, is the Quality of Life in Bronchiectasis Questionnaire (QoL-B). The questionnaire is divided in 8 sections aimed at separately evaluating different aspects of quality of life, named "respiratory symptoms", "physical functioning", "role functioning", vitality, "emotional functioning", "social functioning", "treatment burden" and "health perceptions" [35].

1.6 Exacerbations and the "frequent exacerbator" phenotype

A consensus definition of bronchiectasis exacerbation was published in 2017. Exacerbations are defined by the scientific community as a "Deterioration in three or more of the following key symptoms for at least 48 h: cough; sputum volume and/or consistency; sputum purulence; breathlessness and/or exercise tolerance; fatigue and/or malaise; or haemoptysis AND a clinician determines that a change in bronchiectasis treatment is required"[36]. Exacerbations have a clinical impact in bronchiectasis: frequent exacerbations may lead to disease progression resulting in a worse prognosis. The biological mechanism behind exacerbations is heterogeneous, even if are usually triggered by bacterial infections.

The frequency of exacerbations is a key point in bronchiectasis outcomes. Chalmers and colleagues refer to 3 or more exacerbations/year as the threshold able to predict the risk of future exacerbations, with higher disease severity, lower quality of life, and increased mortality compared to those with a lower number of exacerbations/year [37]. In another study, Araujo *et al.* identified a subgroup with 2 or less exacerbations with an increased mortality, even more increased by *P. aeruginosa* chronic colonization [38].

1.7 Pathogenesis and physiopathology

Bronchiectasis pathogenesis is complex and multifactorial. The pathogenesis of bronchiectasis was described since 1980s with the vicious cycle hypothesis [39]. The components of this cycle are airway structural damage, mucociliary clearance, infection and inflammation. Each of these components may be the start of the vicious cycle based on the aetiology of the disease. Nowadays, the vicious cycle hypothesis has been updated to a vicious vortex, underlying the variability of entries in the vicious cycle, depending on the heterogeneous aetiologies [40].

For example, in NTM infection, pathogens prime inflammation and structural damages; in PCD, there is a failure in mucociliary frequency increasing the risk of pulmonary infections. In the lower airways of bronchiectasis affected patients, a loss in mucous excretion and an overgrowth of microorganisms may lead to inflammation increase [39]. For many years inflammation in bronchiectasis was referred to as neutrophilic one, even if recent experiences drew attention to Th2 high related bronchiectasis [41].

Once the cycle is started, it is self-sustaining and leads to a perpetuation of the disease condition and progression. Therapeutic strategies in bronchiectasis are based on the disruption of this cycle (e.g. the use of inhaled antibiotics or anti-inflammatory drugs).

1.8 Inflammation

Inflammation is one of the factors leading to disease initiation and progression in bronchiectasis. The impaired mucus clearance and chronic bacterial infections may lead to a serious neutrophilic activation by the release of chemotactic mediators. A recent study demonstrated that an increase of sputum neutrophils in bronchiectasis patients was correlated with worse pulmonary function, bacterial colonization, and severe diseaseconditions [42]. Moreover, inflammation levels may cause an increased mucous production and ciliary dysfunction, with a clearance deficiency and increased exposure of the organism to oxidative stress and local infections.

1.8.1 Serine proteases

Serine proteases (NSP) are proteolytic enzymes stored in azurophils granules. These proteins are part of the neutrophilic response to inflammatory triggers, mostly in gram-negatives infection. Neutrophil elastase, cathepsin G and proteinase 3 are synthetized during the granulocyte development in an inactive pre-protein and activated by cathepsin C (dipeptidil peptidasi I – DPPI). DPPI was identified as target for treatment and drugs aiming at inhibiting this protein are nowadays in clinical trial. NSP has both an extracellular and intracellular activity. Inside neutrophils NSP and reactive oxygen species (ROS) are able to work synergically degrading microorganisms trapped in phagolysosomes [43]. NSP are also released in active form, binding and trimming bacterial flagellin, depolarizing bacterial membrane and inhibiting protein synthesis, governing inflammatory cytokines and growth factors through proteolytic activity, cleaving adhesion molecules and contributing to lymphocytes activation. (71-73). Neutrophil elastase (NE) is the most abundant and studied serine protease.

1.8.1.1 Neutrophil elastase

NE is a known biomarker for bronchiectasis, being associated with disease severity, exacerbations, poor quality of life and poor outcomes prediction [44]. At molecular level, experiences in mice with *P. aeruginosa* induced pneumonia proved that the absence of NE was associated with decreased levels of the pro-inflammatory cytokines TNFα, MIP2, and IL-6 in the lungs and with increased mortality of mutant mice due to infection. NE in association with cytokine expression modulation contributes to host protection against *P. aeruginosa* [43]. Furthermore, neutrophils half-life in blood is increase during inflammation. This should lead to functional changes and contributes to the inflammatory-associated injury in bronchiectasis patients [42, 45].

Increased production NE and serine proteases in general can cause extracellular matrix destruction, decreases ciliary beating and promotes expression of mucin, resulting in muco-ciliary clearance failure [46]. NE-dependent structural damage leads to irreversible airway dilation and bronchiectasis development. NE can lead to the degradation of lactoferrin, an important anti-microbial glycoprotein as well as of several molecules involved in pathogens opsonization. Moreover, NE can cause degradation of T-cell surface receptors and interfere with the process of antigen presentation by dendriticcells [47].

1.9 Microbiology

Pulmonary infection is part of the vicious circle leading to bronchiectasis. Infections may both cause bronchiectasis (post-infective aetiology) and sustain the events leading to disease progression. Bronchiectasis lungs are more prone to be colonized by bacteria leading to the development of chronic infections [39].

1.9.1 Standard microbiology

Standard microbiology (bacterial cultures) shows that 80% of patients hosts pathogens in the lungs. The most frequently isolated bacteria are *Pseudomonasaeruginosa* and *Haemophilus influenzae*, *Moraxella catarrhalis*, *Escherichia* species, *Klebsiella* species (Gram-negative) and Gram-positive *Streptococcus pneumoniae* and *Staphylococcus aureus* [31]. Heterogeneity is also present in bacterial infections. Although *H. influenzae* and *P. aeruginosa* are the most common organisms identified in European studies, the US bronchiectasis registry reported *P. aeruginosa* in up to 33% of the patients, while *H. influenzae*was labelled as uncommon (8%) [48]. Patients with a chronic infection by *P. aeruginosa* show higher disease severity, with high frequency of exacerbations, worse quality of life, increased risk of hospital admission and increased mortality [49]. *P. aeruginosa* is a biofilm-forming bacterium. Biofilm protects bacteria from antibiotics and phagocytes, and also produces virulence factors that allow it to evade phagocytes killing and decrease ciliary movement [50–52].

1.9.2 Microbiota

Microbiota can be defined as the complex bacterial community present in a specific ecological niche [53]. Human microbiota has some important functions, for instance development of immunity, defence against pathogens, host nutrition including production of short-chain fatty acids, synthesis of vitamins and fat storage, making it an essential component of the human body [54].

Sputum analysis is an easy and non-invasive method for assessing microbial community in chronic respiratory diseases, although other respiratory samples, such as bronchoalveolar lavage (BAL) or bronchial aspirate (BAS), could be more adequate representatives of the lung flora [55].

Lung is a low nutrient resource for supporting microbiota development compared to the intestinal tract. Physiological conditions are regionally variable even in healthy lungs. Conditions

that affect bacterial proliferation include oxygen tension, blood flow, local pH, temperature, effector inflammatory cell disposition, and epithelial cell architecture [55].

A popular alternative to traditional methods is 16S ribosomal RNA (rRNA) gene sequencing that provides several benefits compared to standard microbiology. 16s rRNA sequencing is fast and relatively inexpensive, often used to determine the composition, abundance and diversity of bacteria and archaea harboured in different ecosystems. DNA sequencing can provide more efficient taxonomic classification than culture-based approaches for many organisms [56].

Microbiota analysis of healthy airways reveals a low abundant community of bacteria [57]. Predominant phyla in healthy lungs are *Bacteroidetes* and *Firmicutes* [58, 59]. *Proteobacteria*-dominated microbiota in bronchiectasis patients was associated with increased neutrophilic inflammation and exacerbations. In contrast, patients with *Firmicutes*-dominated microbiota showed lower neutrophilic inflammation and less frequent exacerbations [60].

It was observed that dysbiosis is typical of chronic respiratory diseases. Measures of richness and evenness, or composite diversity measures such as the Shannon diversity index have a positive linear correlation with lung function in bronchiectasis [61].

Genera that were reported to colonize bronchiectasis lungs during stable state are *Haemophilus*, *Neisseria, Streptococcus, Pseudomonas, Veillonella, Prevotella, Rothia, Klebsiella, Fusobacterium, Porphyromonas, Actinobacillus, Staphylococcus* and *Leptotrichia* [62, 63].

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Generation of the problem and aim of the PhD

Bronchiectasis is a heterogeneous chronic respiratory disease from many points of view including patients' characteristics, causes and biological mechanisms. In order to face this heterogeneity, the adoption of a precision medicine approach is endorsed in bronchiectasis. The identification of biomarkers for patients' stratification, outcome prediction and identification of new targets of treatment is very important in precision medicine and nowadays crucial for the efforts of the scientific community. Microbiome and its interaction with host defences plays a central role in bronchiectasis. Microbiome is central in shaping the pulmonary immune response and dysregulation at microbiota level, leading to pulmonary dysbiosis, that is frequent in respiratory diseases. Although studies aimed at describing microbiome in bronchiectasis has been published, there is a need for further investigations.

The aim of this PhD was to characterize adult patients with bronchiectasis in stable state through the study of both the sputum microbiome and the interaction of sputum microbiome with the local host inflammatory response. The deep understanding of these components may lead to advances to the understanding of bronchiectasis physiopathology and to the identification of new treatable traits and biomarkers in bronchiectasis. The study of sputum microbiome and inflammation in this disease will be addressed from a different side in each chapter.

Specific research questions and specific studies were developed in order to address this general aim, as follows:

1) **Technical development:**A) Our matrix of interest was sputum. Sputum collection is a non-invasive technique that gave us the chance to collect biological material from a relatively high number of patients and is currently used in standard microbiology laboratories for microbiological test of pathogens in bronchiectasis. For this reason, the first study we carried out was aimed at developing an effective technique for extracting and sequencing bacterial DNA from bronchiectasis sputum (Chapter 2.1).

B) Active neutrophil elastase is a well-known biomarker for bronchiectasis patients' conditions and long-term outcomes, however new techniques for the detection of this biomarker were available. The second specific aim of this thesis was to focus on active neutrophil elastase detection in order to identify the most suitable method to measure this biomarker in both CF and bronchiectasis (Chapter 2.2).

2) **Patients' characteristics and sputum microbiome:** A) First, we selected a group of variables that may be associated to microbiome and analysed it with the aim of describing clinical characteristics associated with low microbiome diversity in sputum of adults with bronchiectasis (Chapter 3.1).

B) Then we focused on aetiologies. PCD was associated with microbiome diversity from the previous study. We decided to focus on this aetiology with the aim of describing sputum microbiome along with local inflammation in patients with PCD compared to a group of idiopathic and post infective (Chapter 3.2.1).

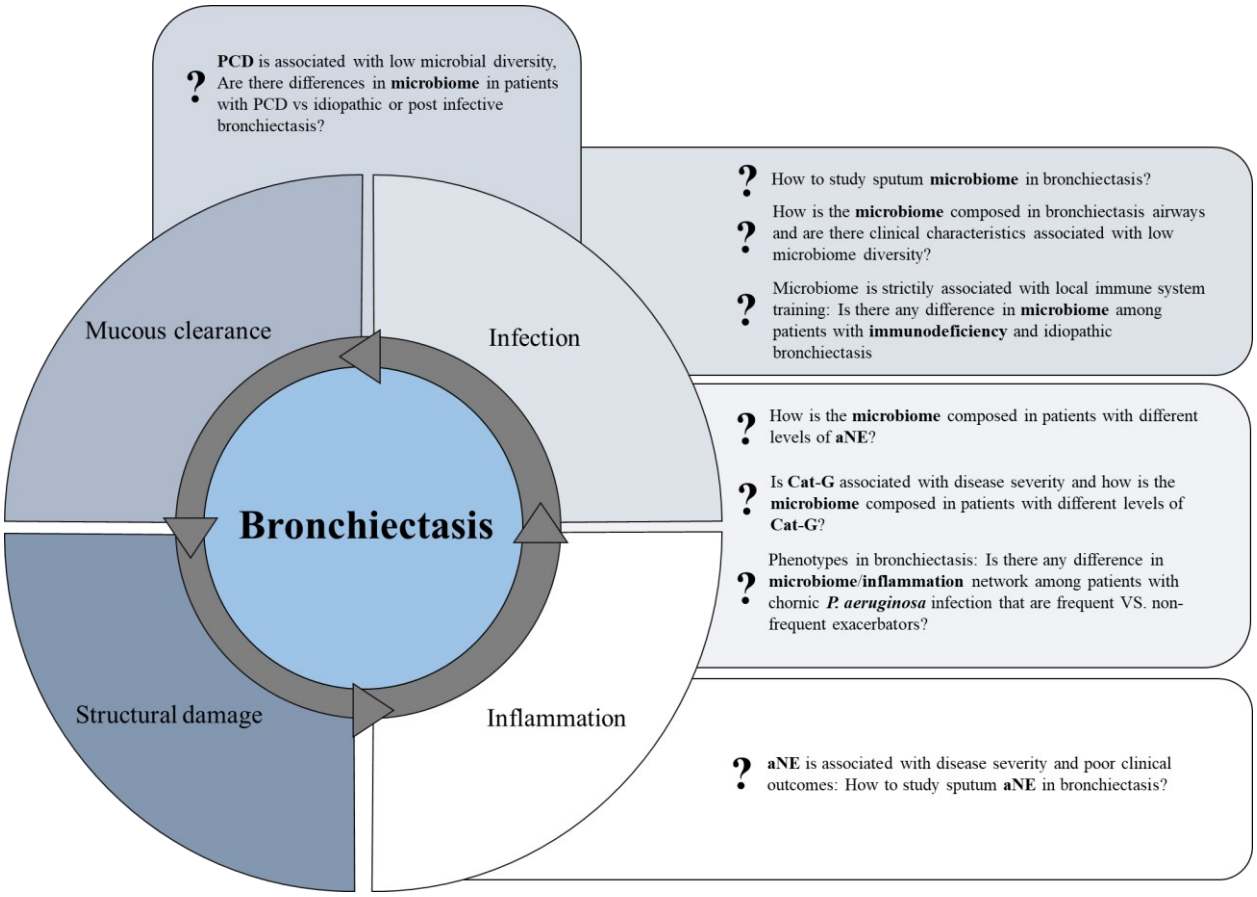
C) Moreover, scientific literature reported lung microbiome as associated with the development of local immune response. Immunodeficiency was an interesting aetiology from this point of view, so we investigated the association between immunodeficiency as an aetiology of bronchiectasis, microbiome and local inflammation (Chapter 3.2.2).

3) **Airway neutrophilic inflammation and microbiome in bronchiectasis**: A) We focused on lung inflammation and specifically on serine proteases. The third specific aim of this thesis was to understand if patients with shared aNE levels shared a peculiar microbiome pattern (Chapter 4.1).

B) The following step was to investigate other inflammatory biomarkers that should be effective in predicting patients' status and outcomes, thus we identified cathepsin G, another serine protease as an interesting protein. The fourth specific aim of this thesis was to investigate the association of cathepsin G with sputum and to compare the performance of both neutrophil elastase and cathepsin G in predicting disease severity, exacerbations and chronic infection with neutrophil elastase (Chapter 4.2).

4) **Sputum microbiome, inflammation and clinical phenotypes in bronchiectasis:** Finally, we focus on a specific group of patients. *P. aeruginosa* chronic infection is one of the most dangerous clinical signals; however, there is a heterogeneity in patients with this chronic infection. Previous works focused on *P. aeruginosa* chronic infection and exacerbations frequency identifying patient with more than two exacerbations/year as more suitable to have worse outcomes. For this reason, the last specific aim of this thesis was to understand the microbiome composition in patients with chronic *P. aeruginosa* experiencing less than two or two or more exacerbations/year and the relationship among microbial community and inflammation in patients' lungs (Chapter 5).

Thesis outline



Chapter 2

2 Technical development

2.1 Comparison of different conditions for DNA extraction in sputum - A pilot study

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2.1.1 Introduction

Respiratory microbiome is a topic of great interest nowadays in translational research for chronic respiratory diseases [1]. Most of the published experiences on respiratory microbiome enrolled chronic obstructive pulmonary disease (COPD) and bronchiectasis patients, including those with cystic fibrosis, and sputum has been the matrix most commonly used by investigators [2–5]. Sputum collection represents an easy and non-invasive strategy for studies on respiratory microbiome. So far, sequencing of microbial communities from airway samples through meta-omic approaches, mainly based on high-throughput DNA sequencing techniques, has been confined to scientific research. Although the use of respiratory microbiome analysis has not been implemented in clinical practice yet, a possible role of this technique in stratifying patients for disease severity and predicting clinical outcomes could be considered [6].

The choice of an appropriate methodology for evaluating the microbiome in sputum samples is crucial to identify the largest biodiversity as possible and to obtain reliable and comparable results. It is a precocious phase for the development of DNA extraction methods from sputum and there is an extreme heterogeneity of DNA extraction technique in literature. However, in this respect, several challenges could be identified. First, sputum is a substrate with a complex and very difficult-to-manage matrix. The use of different techniques for DNA extraction from sputum might be limited by its nature. In order to facilitate its treatment, a solubilizing agent, such as dithiothreitol (DTT), is usually used. [7] Second, epidemiological data on chronic respiratory infections in bronchiectasis and cystic fibrosis revealed the presence of hard-to-lyse bacteria, such as *Staphylococcus aureus,* leading to the possible need of an individualized strategy to enhance bacterial lysis, such as the use of enzymes able to specifically target Gram-positive bacteria [8]. Pre-treatments with lysostaphin and lysozyme, which are able to target cell wall peptidoglycan and pentaglycine bridges respectively, have been reported in literature for microbiome analysis. [9,10] Third, sputum complex and viscous matrix might represent a limitation for broadly used DNA extraction techniques. Among them, chemical coupled with enzymatic lysis is extensively used to treat biological fluids and tissues, while mechanical disruption is usually suggested for soil or feces. Fourth, the bioinformatic approach is not standardized and several pipelines are constantly emerging that can be applied to the analysis of microbiome data generated using next-generation sequencing (NGS) techniques. Finally, largely different methodologies, including steps ranging from wet laboratory practices to *in silico* data analysis, have been published in literature, limiting the comparability of results across different experiences [2,5,11]. It therefore important to assess performance of different methods that can

be applied to the analysis of microbiome in sputum samples with a special focus on the use of DTT and lysostaphin.

The objective of this pilot study was to compare different conditions, such as the use of DTT as a homogenizing agent, lytic enzymes in order to specifically target Gram-positives and DNA extraction technique in order to isolate bacterial DNA from sputum in adult patients with bronchiectasis, according to different endpoints from total DNA extraction to microbiome analysis.

2.1.2 Material and Methods

Five spontaneous sputum samples were collected from five adult bronchiectasis patients followed at the Bronchiectasis Program of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, in January 2018. Subjects signed an informed consent and gave their approval for using samples for the purpose of this study. Aliquots of at least 5 ml of sputum were collected and stored at -80°C for all subsequent analyses.

2.1.2.1 DNA extraction

Eighteen different DNA extraction methodswere evaluated. Three different commercial kits for DNA extraction were considered: 1) Roche High Pure PCR Template Preparation Kit (Hoffmann – La Roche. Basilea. Switzerland); 2) Zymo Quick-DNA Universal Kit (Zymo. Irvine. CA. USA); and 3) Mobio PowerLyzer PowerSoil DNA isolation kit (Mobio. Loker Ave West. Carlsbad. CA. USA). Roche and Zymo use the combination of chemical and enzyme-based lysis, while Mobio a mechanical destruction with bead beating. Commercial kits were used according to manufacturer's instructions. 0.1 g from each sample was extracted with the three kits in duplicate and eluted in 50µl elution buffer.

Two types of pre-treatments of the sputum samples, preceding the DNA extraction itself, were considered: the addition of dithiothreitol (DTT; Sputafluid, Biolife Italiana Srl, Italy) 10% 1:1 in volume to 0.1 g of sputum plugs and the enzymatic digestion with a combination of lysozyme at 3.6 mg/ml and lysostaphin, (at both 0.18 and 0.36 mg/ml) (Sigma-Aldrich. Saint Louis. Missouri, USA). The following six combinations of pre-treatments were performed before using each kit: a) DTT without enzymatic step; b) DTT with 3.6 mg/ml lysozyme and 0.18 mg/ml lysostaphin; c) DTT with 3.6 mg/ml lysozyme and 0.36 mg/ml lysostaphin; d) without DTT and enzymatic step; e) without DTT but with 3.6 mg/ml lysozyme and 0.18 mg/ml lysostaphin; f) without DTT with 3.6 mg/ml lysozyme and 0.36 mg/ml lysostaphin. Samples were incubated at 37° for 30 minutes before DNA extraction [10].

DNA extraction yield for each of the eighteen conditions was measured through quantification by Quant-IT dsDNA Assay Kit. High Sensitivity and Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Subsequently, samples were diluted at 5ng/µl and tested in Real Time PCR using syber green for 16S rRNA gene amplification [12]. Each sample was tested in duplicate and cycle threshold (CT) mean and the standard deviation was considered. Real-Time PCR for *S. aureus*was conducted on DNA extracts to ensure that the addition of lysostaphin is a useful strategy to better lyse and recover the genomic DNA from *Staphylococci*[13].

2.1.2.2 Microbiome evaluation

The V3-V4 variable regions of the 16S rRNA gene were amplified from DNA extracts using the 16S metagenomic sequencing library preparation protocol (Illumina, San Diego, CA, USA). PCR products, approximately sized 460 base pairs, were visualized using microfluidics-based gel electrophoresis on Bioanalyzer 2100 (Agilent Santa Clara, CA, USA) and then were cleaned using AMPure XP magnetic bead-based purification (Beckman Coulter, Brea. CA, USA). Sample libraries were quantified using the Qubit as reported above and then pooled in an equimolar mode. Finally, pool was sequenced on the MiSeq (Illumina, San Diego, CA, USA) sequencing platform, using a 2 x 300 cycle V3 kit and following standard Illumina sequencing protocols.

2.1.2.3 Bioinformatic analysis

Demultiplexed paired-end reads in FASTQ format were received from the Illumina MiSeq instrument. Sequencing data were processed following the UPARSE pipeline by Robert C. Edgar [14], using USEARCH v10.0.240 [15] and VSEARCH v2.3.4 [16]. Overall run quality was checked using FastQC v0.11.2 [17] and reports were summarized using MultiQC v1.4 [18]. Quality scores dropped towards the end of the reverse reads, so they were globally trimmed at position 200 before merging with the corresponding forward reads. Parameters for paired-end reads merging were set as follows: minimum overlapping length 19 base pairs; minimum 90% identity of alignment; merged sequences length restricted to 430-480 bases. Consensus sequences from all samples were pooled together and primers were stripped from both ends. This "raw" set of merged sequences was then quality-filtered and de-replicated to obtain a subset of high-quality unique sequences to be clustered into Operational Taxonomic Units (OTUs). Sequences with more than 1 expected number of errors (EE) were discarded and singletons removed during de-replication. OTUs were clustered at 97% identity threshold. Taxonomy prediction to the genus level for OTU sequences was performed via the SINTAX algorithm [19], using the RDP training set v16 as reference database and 0.8 as confidence threshold. An OTU table was constructed by mapping the whole set of "raw" merged paired-end reads to the representative set of OTUs, using 97% identity threshold. It was then filtered - low abundance OTUs (< 0.5 overall frequency) discarded - and normalized to the same number of reads per sample. This OTU table was used for all downstream analyses. Alpha diversity was measured for each sample using different metrics (Shannon entropy and Simpson estimators). These indices were then converted to effective number of species (ENOS) [20]to be easily compared to each other.

2.1.2.4 Study endpoints and statistical analysis

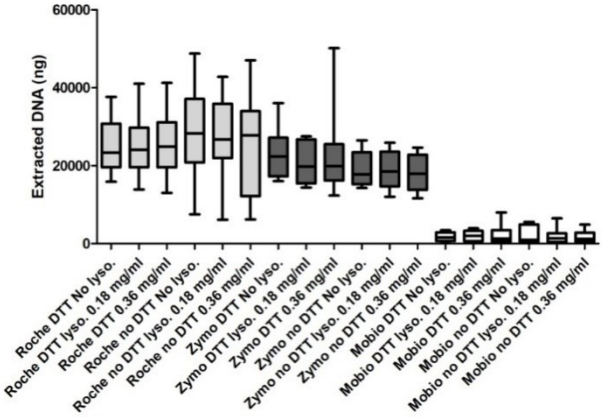
The following results have been compared across the 18 different methods: Extracted DNA yield, Real-time PCR for 16s rRNA gene, Diversity indices, including Shannon entropy and Simpson estimators and their conversion into ENOS. Relative abundances and Real-time PCR for *S. aureus*. Relative abundances have been compared with results of standard microbiology.

One-way ANOVA with post hoc Bonferroni test has been conducted on Real-Time PCR data with a significance level of 0.05. Statistical analysis was performed in the R environment.

2.1.3 Results

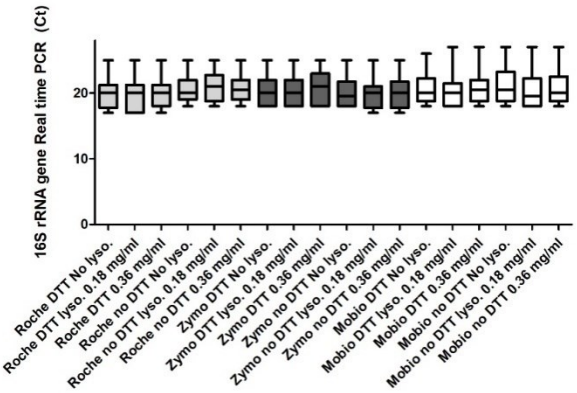
DNA was recovered from all the five sputum samples. A higher quantity of DNA has been extracted using Roche and Zymo kits in comparison to Mobio one; the median values of extracted DNA across the 6 different conditions for Roche, Zymo and Mobio kits were 26.062, 19.750 and 1.233 ug respectively. Detailed median (IQR) values of extracted DNA for all the 18 different conditions are reported in Figure 1.

Figure 1. Comparison between median (IQR) levels of DNA extraction yield across 18 evaluated conditions.



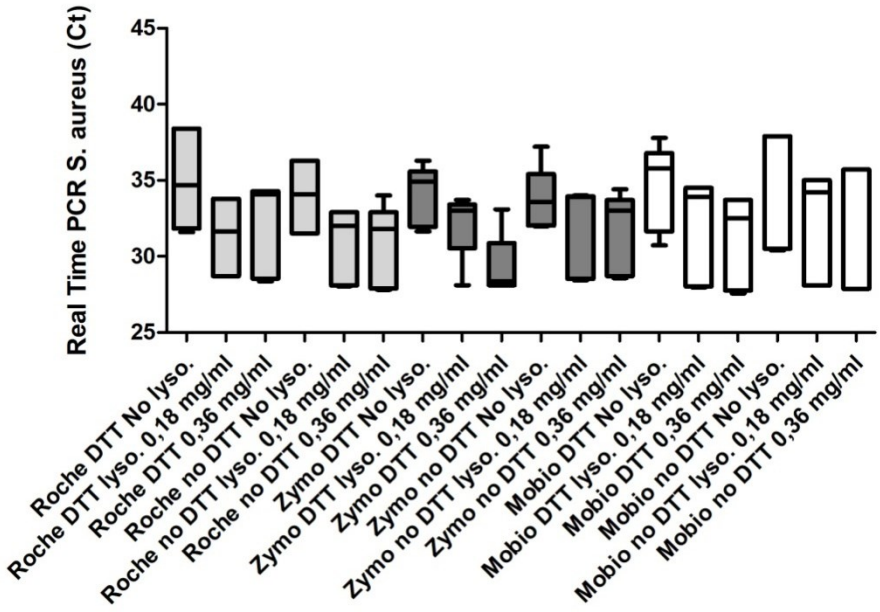
Results of the real-time PCR for 16S rRNA gene for each condition (Figure 2), show that no significant differences across the 18 conditions (range of medians between 20 and 21 Ct).

Figure 2. Comparison between median (IQR) levels of 16s rRNA gene Real-Time PCR across 18 evaluated conditions.



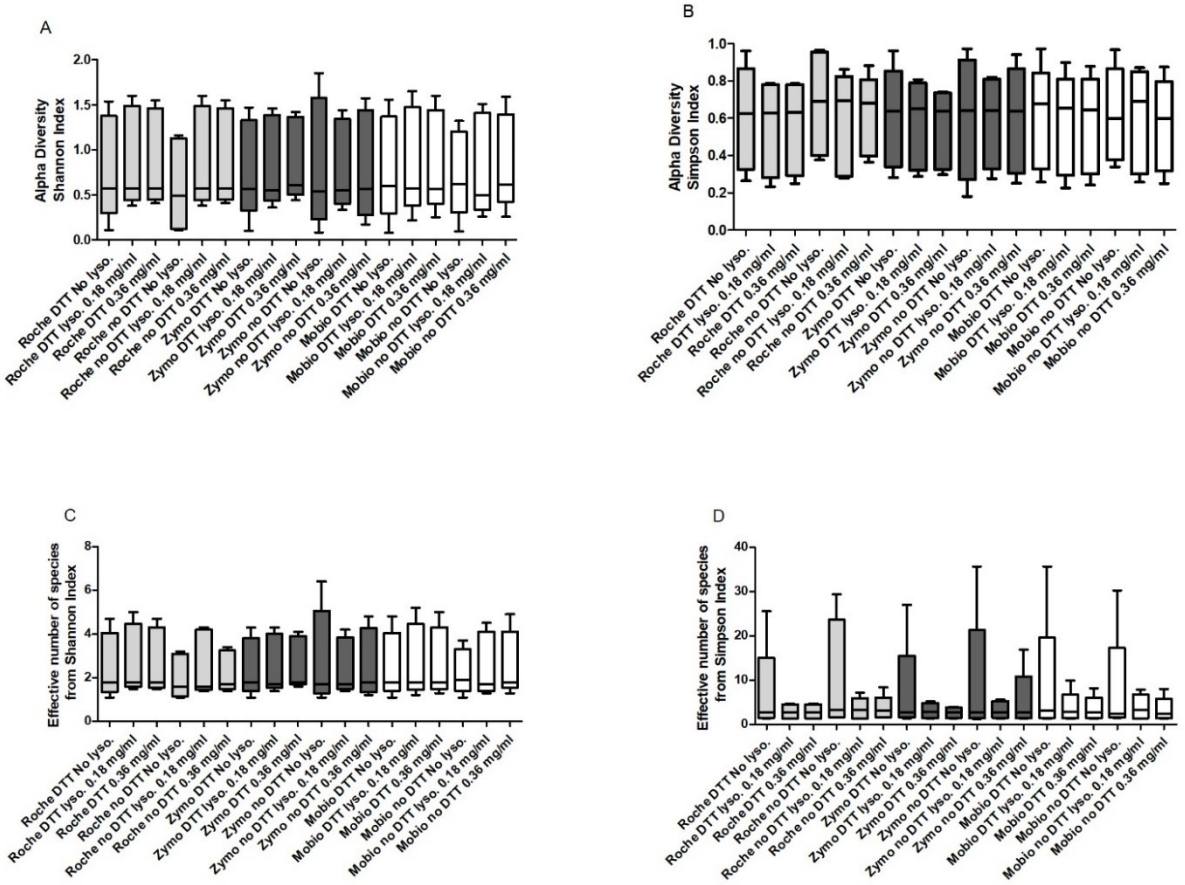
Real-time PCR for *S. aureus*has been performed for all samples and was positive for two of them. The addition of the enzymatic digestion step increased *Staphylococcus* DNA extraction (Figure 3). A similar pattern can be observed through all the conditions even if no statistical evidence is present.

Figure 3. Comparison between median levels (IQR) of Real-Time PCR for S. aureus in sputum samples and across the 18 evaluated conditions



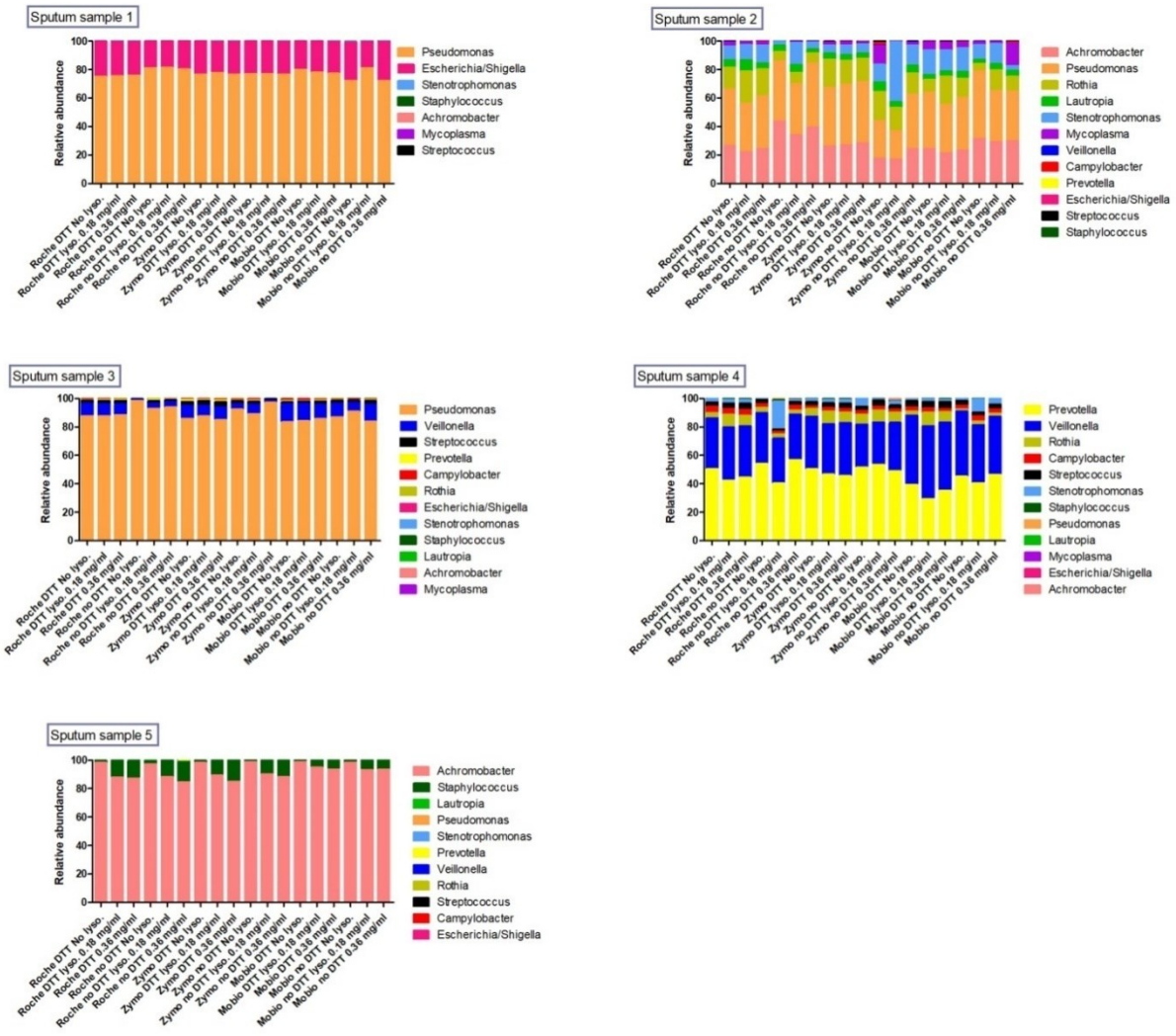
In terms of alpha diversity, no significant differences have been detected among the 18 evaluated conditions, neither considering the Shannon index (range of median values: 0.492-0.574), nor the Simpson (range of median values: 0.624-0.692). Evaluation made using ENOS from Shannon index (range of median values: 1.6-1.8) and ENOS from Simpson index (range of median values: 2.7-3.3), did not show any appreciable difference (Figure 4).

Figure 4. Comparison of median levels of Alpha diversity across the 18 evaluated conditions expressed as Shannon index and relative effective number of species (ENOS) (3A and 3B), and Simpson index and relative ENOS (3C and 3D)



Relative abundances at the genus level of identified bacteria in each sputum sample across the 18 evaluated conditions are reported in Figure 5**.** Microbiome composition is very different across different samples. Sputum samples presenting few genera have very similar data, while in the presence of a higher number of bacterial genera (Patient 2 and 4) differences emerge when comparing the same condition with and without DTT.In sputum sample 5, where only two genera are present, there is an increase of *Staphylococci* presence in samples treated with lysostaphin and lysozyme.

Figure 5. Relative abundances of bacterial genera in each of the 5 sputum samples across the 18 evaluated conditions



2.1.4 Discussion

The major finding of the present experience is that the 18 different conditions evaluated seem to equally perform in extracting DNA from sputum samples in terms of Real-Time PCR for 16s rRNA gene, alpha diversity and relative abundances of bacterial genera. However, the DNA extraction yield seems to be higher if Roche and Zymo kits are used in comparison to the Mobio one.

The use of enzymatic techniques seems to allow a higher DNA extraction yield than mechanical ones. A high yield might be preferred for 16s rRNA sequencing because sputum is a matrix with a high percentage of human DNA. For this reason, in order to prepare libraries with an acceptable bacterial DNA amount and more representative for the microbiota of the sample, the starting amount of DNA loaded into PCR needs to be high. In order to overcome this limitation, it is possible to either increase the amount of sputum collected by patients or, in case of a limited amount of sputum sample, select a technique able to obtain a high yield of DNA (such as Roche or Zymo kits).

Both the evaluated pre-treatments seem to play a crucial role. The addition of lytic enzymes is important to enhance *S. aureus* DNA extraction; however, an increase of lysostaphin concentration over 0.18 mg/mL seems not to increase the performance of the kit to extract DNA. These results, first identified through Real-Time PCR for *S. aureus*, are confirmed by an increase in *Staphylococcus* genus identification through sequencing. The addition of DTT has a role in improving data reproducibility. Through sequencing, we saw that the presence of DTT seemed to be able to better homogenize sputum samples, leading to more reproducible results in terms of bacterial genera detected, and at the same time seems not to affect the efficiency of DNA extraction. Relative abundances in sputa presenting a large number of bacterial genera are very similar in conditions with DTT, while they vary greatly but very different when it is absent. In sputum sample 2 there are differences in extraction of *Stenotrophomonas* genus, greatly improved in absence of DTT, presence of 0.18 mg/ml lysostaphin and treated with Zymo kit. Comparing this result with the same condition, in presence of DTT we might speculate that the difference is given by sampling. DTT enable the release of bacteria entrapped into sputum matrix. For this reason, the repetition of the analysis on the same sample leads to comparable results. If a homogenization step is not present, differences given by sampling can be present.

This study has some limitations. First, different interventions, such as the use of DTT and lysostaphin, have been performed at the same time, while no sequential approach has been used.

Although this might help us in evaluating possible synergies between variables, difficulties in data interpretation might be present without a special focus on a single variable. Second, the monocentric design, the low number of samples collected and the fact that no patients other than bronchiectasis have been enrolled might interfere with the generalizability of our results. Third, only three kits have been considered, although others are on the market. Finally, our study did not take into account the possible presence of initial contamination of commercial kits.

This is the first pilot study that tried to address relevant methodological questions in microbiome analysis of sputum samples and took into consideration not only a comparison among different commercial kits but also different pre-treatments of sputum samples. Furthermore, the performance of 18 different conditions for DNA extraction from sputum has been evaluated considering different endpoints. Real-time PCR for both 16s rRNA gene allowed us to understand if all methods were able to extract bacterial DNA with the same efficiency; Real-time PCR for *S. aureus* has been performed in order to evaluate lytic enzymes activity; Microbiome analysis considering alpha diversity indices and relative abundances.

2.1.5 Conclusions

The use of a *unique* method for DNA extraction and microbiome analysis of sputum samples is very important in translational research and could represent a step forward in the introduction of microbiome analysis in clinical practice. A homogeneity of methods for microbiome analysis is needed as well as data coming from different centres which are needed to improve the reproducibility of the method. These data should be important for the development of new methods in order to have an improvement of DNA extraction techniques for microbiome analysis.

None of the 18 evaluated conditions seems to be superior to the other ones in extracting DNA from sputum samples, although a higher amount of extracted DNA could be obtained using enzyme-based commercial kits. Pre-treatments with lysostaphin, lysozyme and DTT seem to be necessary in order to have the most representative microbiome evaluation possible. Further studies will be necessary in order to confirm our data. Moreover, these preliminary data show that neither synergic nor interfering effect is present between variables. Punctual evaluation of variables in an independent way should be needed in order to better address this issue. The hypothesis of evaluating microbiome from the same sputum samples in accordance with the same standard operating procedures across different international centreswill be considered in case of multicentre studies on microbiome analysis will be designed in the next future.

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2.2 Evaluation of active neutrophil elastase in sputum of a comparison among different techniques

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2.2.1 Introduction

Bronchiectasis due to either cystic fibrosis (CF) or other genetic/acquired conditions (hereafter described as bronchiectasis) are chronic respiratory diseases significantly affecting patients' quality of life and clinical outcomes [1,2]. Respiratory symptoms are caused by a vicious circle including anatomical changes (bronchiectasis), impaired airway clearance, and dysregulated local immune defence, followed by chronic bacterial infections and neutrophilic inflammation [3–6].

Neutrophils are key to host defence against infection in the lung but excessive neutrophilic inflammation can contribute to tissue damage. Neutrophil elastase (NE) released through degranulation or extracellular trap formation in response to infection, may be harmful if not properly regulated by local inhibitors [7–10]. Several studies have recently demonstrated that NE in sputum is a biomarker of disease severity and predicts clinical outcomes in patients with bronchiectasis and CF [11].

Several assays have been developed to measure active NE in sputum samples and different principles for activity determination have been employed. One of the oldest and most frequently used spectrophotometric method is based on the ability of the protein to cleave a chromogenic or fluorogenic peptide-based substrate. Numerous substrates and reporters, with a large range of specificity, have been used. [12]. Some peptides (e.g., peptide 4-nitroanilides, peptidyl 4-methyl-7 coumarylamides, and naphtylamides) are easy to use but do not to properly discriminate between proteases present in sputum [11,12]. Moreover, activity-based immunoassays have been developed and one of them was recently validated in a large cohort of bronchiectasis patients [6].

The objective of the present study was to compare three different methods, a ProteaseTag® Active NE Immunoassay (ELISA) and two enzymatic digestion assays (chromogenic –CS- and fluorogenic –FS- substrate), for the quantification of active NE in sputum samples collected from CF and bronchiectasis patients, to assess their reproducibility, consistency, and agreement, as well as correlation between NE activity levels and clinical data.

2.2.2 Materials and Methods

This was a prospective observational study conducted at the Bronchiectasis and Cystic Fibrosis Programs of the Respiratory Department of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico in Milan (Italy). Sputum samples from 50 bronchiectasis and 50 CF patients in stable clinical conditions were collected between March 2017 and July 2018. All subjects provided written informed consent to participate. Bronchiectasis patients underwent clinical evaluation including the calculation of the bronchiectasis severity index (BSI) as previous described (BSI >8 was regarded as severe) and sputum colour was evaluated using the Murray colour chart (purulence if Murray scale >4) [13]. In the CF cohort, mucus purulence was identified through colour assessment from trained healthcare professionals as per standard operating procedures. Spontaneous sputum samples were obtained and sputum samples were processed getting first rid of saliva, sputum plugs selected and weighted. Samples were diluted 8X in Phosphate-Buffered Saline (PBS), vortexed until sputum dissolution and centrifuged for 15 minutes at 3,000 g. Sample supernatants were recovered and stored at -80°C.

Three different methods to evaluate NE activity were used (Table 1), including:

ProteaseTag® Active Neutrophil Elastase Immunoassay (ELISA) –Assay 1 which was conducted according to the manufacturer's instructions.

NE activity evaluation through Meosuc-ala-ala-pro-val-AMC digestion (Chromogenic Substrate, CS) –Assay 2-. Samples were evaluated through the analysis of a specific substrate digestion (Meosuc-ala-ala-pro-val-AMC). They were diluted 1:100 in 50mM of Sodium acetate (sigma Aldrich) pH 5.56, and 200mM of NaCl (Sigma Aldrich). A standard curve was prepared using elastase form human leukocytes (Sigma Aldrich) and diluted in 50 mM HEPES pH 8 (Sigma Aldrich) and 150 mM NaCl; CS Meosuc-ala-ala-pro-val-AMC (Sigma Aldrich) was first diluted at 50mM in DMF (Sigma Aldrich) 30% and subsequently diluted 1:25 in samples buffer. Forty uL of samples were incubated with at 37°C with 40ul of CS for 40 minutes with reads every 5 minutes at emission 360nm, excitation 465 nm. Samples were evaluated using TECAN infinite M200Pro (Tecan, Männedorf, Switzerland). Data in linear phase were considered for statistical analysis. [6]

NE activity evaluation through Abz-Ala-Pro-Glu-Glu-Ile-Met-Arg-Arg-Gln-EDDnp digestion (Fluorogenic Substrate, FS) –Assay 3. Samples were evaluated through the analysis of a specific substrate digestion (Abz-Ala-Pro-Glu-Glu-Ile-Met-Arg-Arg-Gln-EDDnp). They were diluted 1:50 in a specific buffer made of 50mM of Sodium acetate (Sigma Aldrich, USA) pH 5.5, and

200mM of NaCl (Sigma Aldrich). A standard curve was prepared using elastase form human leukocytes (Sigma Aldrich, USA) and diluted in 50 mM HEPES pH 7.4 (Sigma Aldrich, USA) and 150 mM NaCl; Fluorogenic Substrate (Peptide institute, Japan) was first diluted at 5mM in DMF (Sigma Aldrich) 30% and subsequently diluted at 1 mM in PBS. 150 uL of samples were incubated at 37°C with 3ul of FS for 45 minutes with reads every 5 minutes at emission 320nm, excitation 420 nm. Samples were evaluated using TECAN infinite M200Pro (Tecan, Männedorf, Switzerland). Data in linear phase were considered for statistical analysis. [11]

Table 1. Assays used to evaluate neutrophil elastase activity for the present study

	ELISA	CS	FS
Technique development	Commercial	In home	In home
Type of evaluation	Quantitative	Quantitative	Quantitative
Principle	Active NE immunoassay	Enzymatic substrate digestion	Enzymatic substrate digestion
Time needed to analyze 40 samples	3 h	45 min	20-50 min
Sample dilution	1:100	1:100	1:50
Sample type	Soluble sputum	Soluble sputum	Soluble sputum
Standard curve range	1000-15.625ng/ml	3000-93,75 ng/ml	750-12 ng/ml

Wilcoxon signed rank test and Friedman test with Dunn's post hoc test for paired analysis were computed. A statistical significance level of <0.05 was considered. Reproducibility was tested comparing replicates. Intraclass correlation (ICC) between two replicates on the same plate was calculated, measuring data consistency and agreement. Spearman correlations between the level of NE detected through each method and patients' clinical characteristics was assessed. Data are presented combined and for the two separated cohorts.

2.2.3 Results

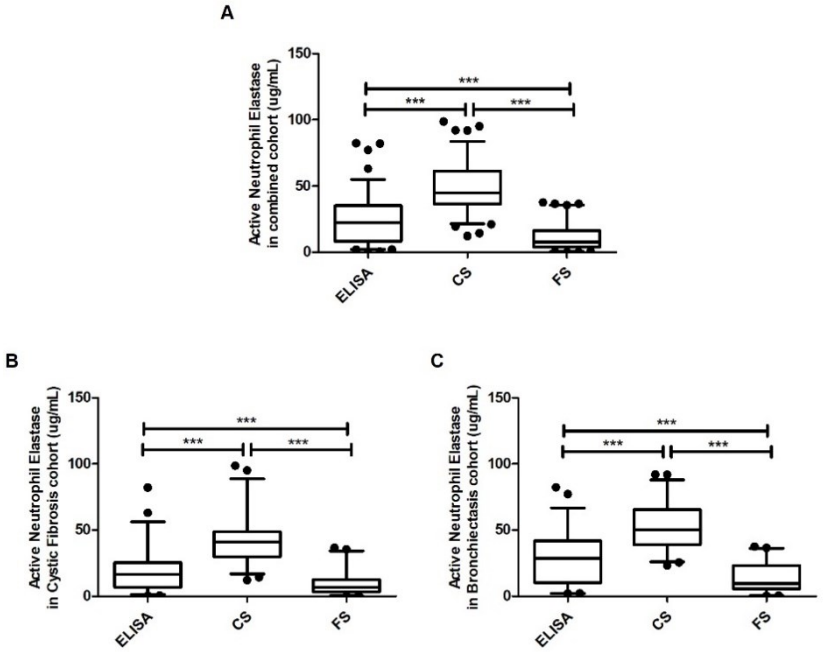
Demographics, clinical, functional, and microbiological data of both CF and bronchiectasis cohorts are showed in Table 2.

Table 2. Descriptive analysis of the CF and bronchiectasis cohorts.

Variables	**Bronchiectasis**	**Cystic Fibrosis**
	n= 50	n= 47
Males, n (%)	11 (22.0)	26 (55.3)
Median (IQR) or Mean (SD) age, years	67 (59-74)	41.2 (8.9)
Mean (SD) or Median (IQR) BMI, kg/m2	21.6 (3.7)	22.3 (21.0-24.2)
Median (IQR) BSI	8 (5-12)	Not applicable
Mean (SD) FEV_1, %predict.	77.5 (26.6)	67.5 (19.3)
Mucopurulent/purulent sputum, n (%)	35 (70.0)	13 (28.9)
Median (IQR) daily sputum volume, mL	20 (6-50)	25 (15-50)
Chronic infection with any bacteria, n (%)	27 (54.0)	45 (95.7)
Chronic *P. aeruginosa* infection, n (%)	20 (40.0)	22 (46.8)

Median (IQR) levels of NE activity were statistically different across the three methods (ELISA, CS and FS) in the combined [ELISA: 22.24 (0.267- 86.26) µg/mL; CS: 44.77 (12.21-98.74) µg/mL; FS: 7.747 (0.0030- 37.55) µg/mL; ELISA vs. CS: P <0.0001; ELISA vs. FS: P <0.0001; CS vs. FS: P <0.0001], CF [ELISA: 16.41 (0.2670-82.02) µg/mL; CS: 40.95 (12.21- 98.74) µg/mL; FS: 6.822 (0.0030-36.61) µg/mL; ELISA vs. CS: P <0.0001; ELISA vs. FS: P <0.0001; CS vs. FS: P <0.0001] and bronchiectasis cohort [ELISA: 28.52 (2.115-82.26) µg/mL; CS: 50.18 (23.18-92.06) µg/mL; FS: 9.620 (0.3070- 37.55) µg/mL; ELISA vs. CS: P <0.0001; ELISA vs. FS: P <0.0001; CS vs. FS: P <0.0001] (Figure 1 and Table A in the online supplement).

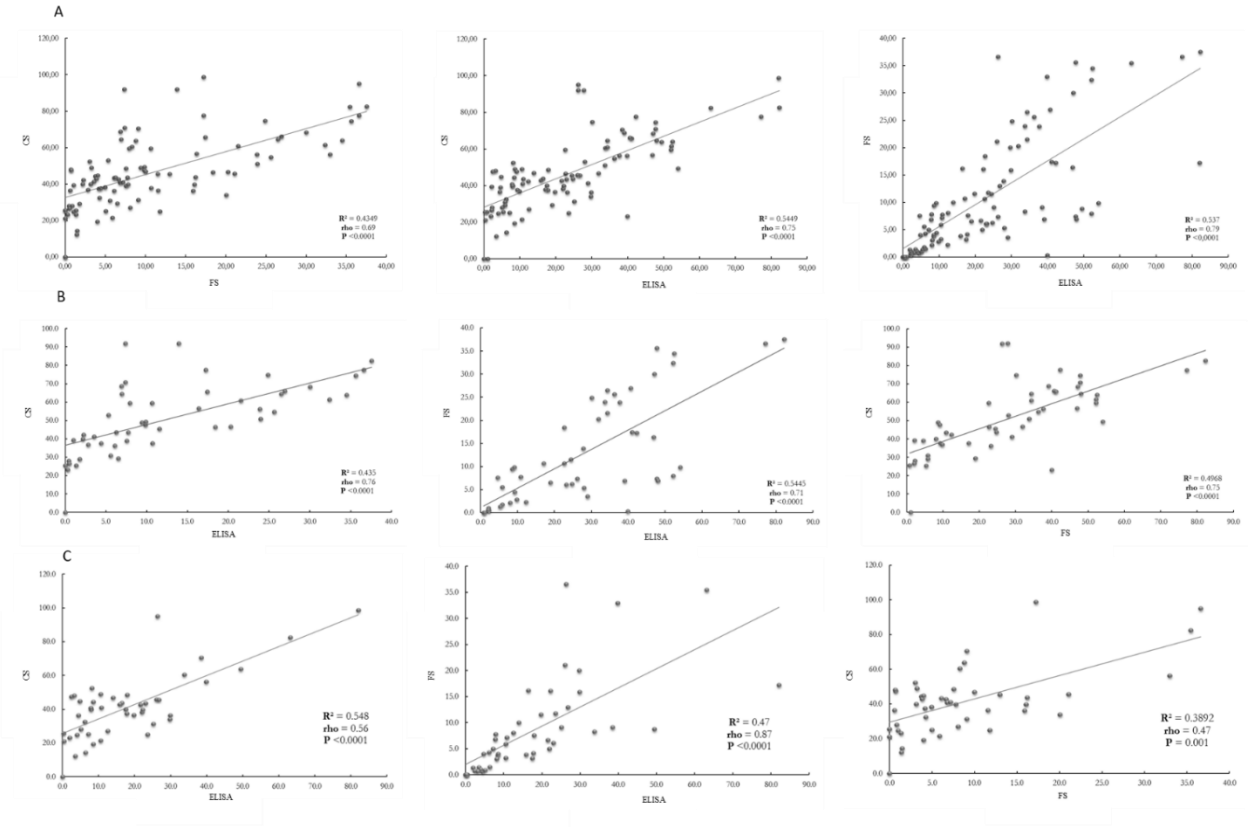
Figure 1. Comparison of median levels of active NE across the three different methods. Friedman test p<0.0001. ***: p<0.0001 Wilcoxon signed rank test



In terms of consistency, the three methods showed similar performance, with FS having the highest ICC (FS 0.9981, ELISA 0.9939, CS 0.9591). Agreement between replicates provided similar results with an ICC of 0.9990 for FS, 0.9969 for ELISA, and 0.9791 for CS.

Correlations on NE activity were reported in Figure 2. The strongest correlation was detected between FS and ELISA (rho= 0.79) for the combined cohort, between FS and CS (rho= 0.76) for the bronchiectasis cohort, and between ELISA and FS (rho= 0.82) for the CF cohort. A good correlation was found among the three methods in all three cohorts.

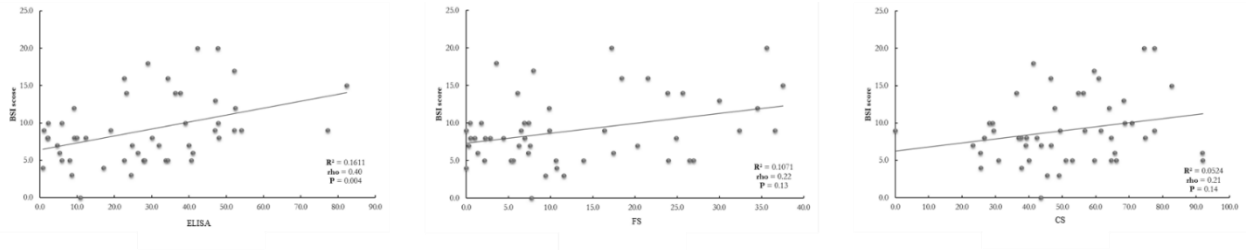
Figure 2. Correlation between results of neutrophil elastase activity measured through the three techniques A) in the combined cohort; B) in the bronchiectasis cohort and C) in the CF cohort.



2.2.3.1 Bronchiectasis Cohort

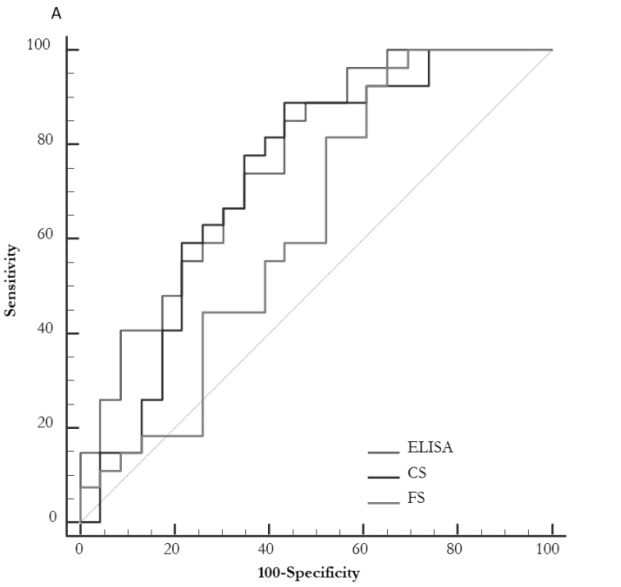
Median (IQR) BSI value in the bronchiectasis cohort was 8 (5-12) and 23 (46%) patients had severe bronchiectasis. The highest correlation (rho= 0.40, P<0.0001) between NE activity in sputum and disease severity (BSI score) was showed with the ELISA method (Figure 3).

Figure 3. Correlation between neutrophil elastase activity measured through the three different methods and the bronchiectasis severity score in the bronchiectasis cohort

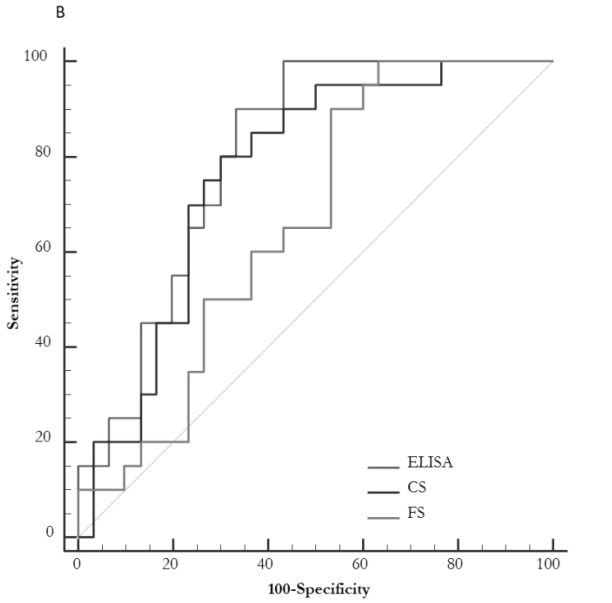


Furthermore, ELISA method showed the highest AUC (0.74; 95%CI:0.65-0.95; FS AUC 0.66; CS AUC 0.62) in predicting severe disease in the bronchiectasis cohort (Figure A in the online supplement). A total of 17 (34%) patients had daily purulent sputum. The performance of NE activity in terms of prediction of sputum purulence was better when the ELISA method was compared with the other two methods (ELISA: AUC= 0.79; CS: AUC= 0.64; FS: AUC= 0.65; Figure B in the online supplement). NE activity measured through both the ELISA and CS techniques could similarly predict chronic infection due to any bacteria (ELISA AUC= 0.76 VS. CS AUC= 0.73; P value between ROCs= 0.5504), whereas FS showed a lower performance (FS AUC= 0.63; FS VS. ELISA: P= 0.0258; FS VS. CS: P= 0.0707) (Figure 4). NE activity measured through both ELISA and CS performed better than FS in predicting chronic *P. aeruginosa* infection, (ELISA: AUC= 0.80; CS: AUC= 0.77; FS: AUC= 0.66; ELISA VS. FS, P= 0.0177; CS VS. FS, P= 0.0887) (Figure 4). No correlation has been found between NE activity and FEV_1% predicted values (FS rho= 0.10; CS rho= 0.00, ELISA rho= -0.04).

Figure 4. Comparison of ROC curves of neutrophil elastase activity measured through different methods in predicting A) any chronic infection and B) chronic *P. aeruginosa* infection in the bronchiectasis cohort



Chronic infection	OR (95% CI)	p-value	AUC (95% CI)
ELISA	1.1 (1.0-1.1)	0.004	0.76 (0.63-0.90)
CS	1.1 (1.0-1.1)	0.01	0.73 (0.58-0.88)
FS	1.0 (1.0-1.1)	0.23	0.63 (0.47-0.79)

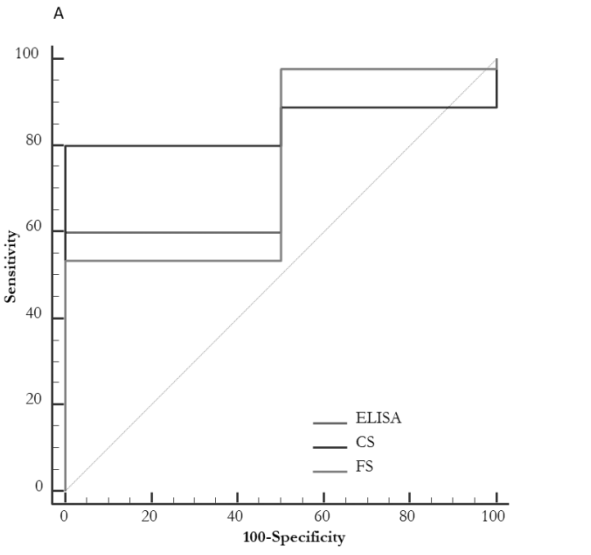


Chronic *P.aeruginosa* infection	OR (95% CI)	p-value	AUC (95% CI)
ELISA	1.1 (1.0-1.1)	0.001	0.80 (0.68-0.92)
CS	1.1(1.0-1.1)	0.006	0.77 (0.63-0.90)
FS	1.0 (1.0-1.1)	0.13	0.66 (0.51-0.81)

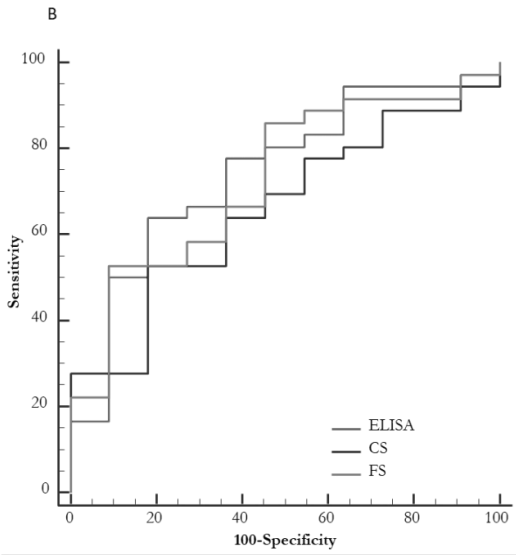
2.2.3.2 Cystic Fibrosis Cohort

Median (IQR) sputum volume of the CF cohort was 25 (15-50) mL/day. NE activity measured through the three techniques performed similarly in predicting sputum quantity in the CF cohort (CS: rho= 0.67; ELISA: rho= 0.69; FS: rho= 0.71) (Figure E in the online supplement). No statistically significant differences were found in terms of NE activity as predictor of any chronic bacterial infection among the three methods (Figure 5). CS was the method with the highest performance (AUC= 0.84), whereas ELISA showed an AUC of 0.79 and FS an AUC of 0.76. Moreover, NE activity measured through FS and ELISA performed similarly in predicting chronic *P. aeruginosa* infection. The highest value of AUC was detected with the ELISA method (AUC= 0.75), whereas FS and CS showed an AUC of 0.73 and 0.66 respectively (Figure 5). The highest correlation between active NE and FEV_1% was found with FS (rho= 0.42, P=0.03) in comparison with the other methods (ELISA rho= 0.36; CS rho= 0.24).

Figure 5. Comparison of ROC curves of neutrophil elastase activity measured through three different methods and chronic infection A) with any bacteria, B) with *P. aeruginosa* in the CF cohort



Chronic Infection	OR (95% CI)	p-value	AUC (95% CI)
ELISA	1.1 (1.0-1.1)	0.004	0.79 (0.65-0.89)
CS	1.0 (1.0-1.1)	0.01	0.84 (0.71-0.93)
FS	1.0 (1.0-1.1)	0.23	0.76 (0.61-0.87)



Chronic *P. aeruginosa* infection	OR (95% CI)	p-value	AUC (95% CI)
ELISA	1.1 (1.0-1.2)	0.04	0.75 (0.58-0.91)
CS	1.0 (1.0-1.1)	0.12	0.66 (0.49-0.83)
FS	1.2 (1.0-1.4)	0.07	0.73 (0.56-0.90)

2.2.4 Discussion

This study shows that levels of NE activity in sputum, as well as their correlations with clinical, functional, and microbiological characteristics, might vary according to the diagnostic method in bronchiectasis and CF patients.

The first evidence was that levels of NE activity were statistically different across the three methods. These method-related NE activity level differences could be explained by the method specificity and discrimination ability between serine proteases. CS is the oldest method and is considered to be less specific compared to FS. ELISA takes advantage of a different principle based on ProteaseTags®, which have the capacity to rapidly and selectively bind active proteases such as elastase with a high level of specificity and selectivity. Based on that, we could speculate that the most specific technique shows the lowest median levels of NE activity, whereas the less specific technique could not adequately discriminate between the action of serine-proteases, providing the highest activity levels. Selection of the appropriate method for NE activity measurement in sputum samples is key: scientists and clinicians should be aware of the different performances. From a research point of view, it might be used as a biomarker in early phase clinical trials. In the future, the evaluation of NE activity might also be implemented in clinical practice as a point of care for outcome prediction. Moreover, it could be used in clinical trials as well as for patients' stratification and outcome prediction.

Our study demonstrated that the explored techniques to measure NE activity show different correlation values between biomarker levels and clinical, microbiological, and functional characteristics in both CF and bronchiectasis patients. An elevated correlation between NE activity and disease severity, sputum purulence, and chronic infections due to both any pathogen and *P. aeruginosa* was found in bronchiectasis patients with the strongest correlations observed with the activity-based immunoassay, which seems to be the most suitable method for the BE population. In the CF cohort a good correlation between NE activity levels and sputum quantity, chronic infection and FEV_1% was observed mainly when FS method was utilized. Nevertheless, the correlation values observed with the FS method were mostly close to those obtained with the activity-based immunoassay, suggesting a more appropriateness of these two techniques compared to the CS method for the CF cohort. Although we recognize the limited statistical power of our study in view of the small sample size, we could speculate that in bronchiectasis patients NE activity levels showed better correlation with clinical data using the activity-based immunoassay probably because this method was specifically developed for bronchiectasis patients and its validation in a large bronchiectasis cohort showed good results [6].

Our findings on the correlation between active NE measured with the ELISA method and BSI are similar to those recently published by Chalmers and coworkers (with rho of 0.49 and 0.40, respectively) [6]. No correlation with between active NE and FEV_1% was found in our cohort in contrast with published data. This may be related to the lower sample size in our cohort in comparison or the different clinical factors which can affect lung function in bronchiectasis patients.

We found a correlation between NE activity measured through the ELISA kit and sputum microbiology. Chalmers and colleagues identified an increase in NE levels in patients chronically infected with *P. aeruginosa*, *Enterobacteriaceae*, and *H. influenzae*, and our data on chronic infections seem to confirm those published in the Scottish experience [6].

The choice of the best assay to evaluate NE activity in sputum samples for patients with chronic respiratory diseases might have important implications. From a research perspective, the comparison of results across different studies on NE activity should take the method in consideration. Furthermore, if levels of NE activity in sputum will be used as an inclusion criterion for future experimental studies on NE-inhibitors, the choice and the standardization of the technique used to evaluate the biomarker might have important consequences. From a clinical perspective, if NE activity will be used to assess disease severity and the use of specific treatments in both bronchiectasis and CF patients, the findings of the present study could be helpful to select the most adequate methodology. [14]

However, several limitations can be found, from the single-centre design to the small sample size. This study is one of the first experiences comparing different available methods for measuring active NE in sputum and to evaluate clinical, functional, and microbiological data in two different patient population groups.

2.2.5 Conclusions

NE activity in sputum correlates with clinical variables in both CF and bronchiectasis. However, available techniques for active NE measurement in sputum can show different results, with different correlations with clinical data. The choice of the most suitable technique might depend on the disease where NE activity levels should be analysed.

2.2.6 References

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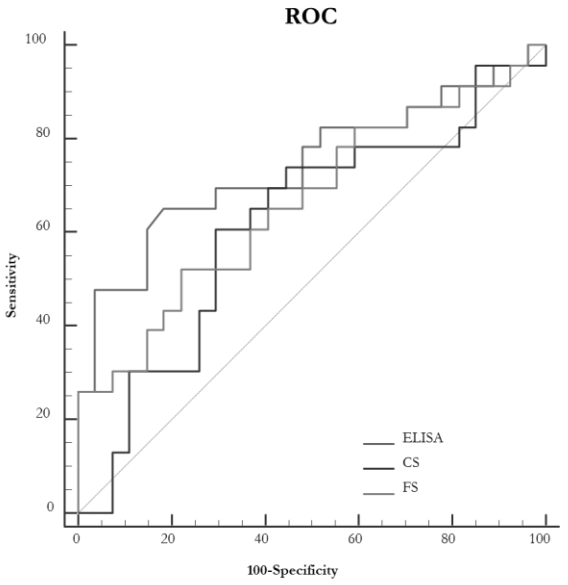
2.2.7 Online supplement

Table A. Descriptive statistics within the three cohorts

Combined cohort			
	ELISA	CS	FS
Median (µg/mL)	22.24	44.77	7.747
Minimum (µg/mL)	0.2670	12.21	0.0030
Maximum (µg/mL)	82.26	98.74	37.55
25% Percentile (µg/mL)	8.293	36.37	3.814
75% Percentile (µg/mL)	35.23	61.19	16.29
CF cohort			
	ELISA	CS	FS
Median (µg/mL)	16.41	40.95	6.822
Minimum (µg/mL)	0.2670	12.21	0.0030
Maximum (µg/mL)	82.02	98.74	36.61
25% Percentile (µg/mL)	6.738	29.75	3.225
75% Percentile (µg/mL)	25.56	48.80	12.40
Bronchiectasis cohort			
	ELISA	CS	FS
Median (µg/mL)	28.52	50.18	9.620
Minimum (µg/mL)	2.115	23.18	0.3070
Maximum (µg/mL)	82.26	92.06	37.55
25% Percentile (µg/mL)	10.13	39.12	5.393
75% Percentile (µg/mL)	41.98	65.43	23.29

Figure A. Comparison of ROC curves of NE activity evaluated through the three methods in predicting disease severity (BSI) in the bronchiectasis cohort



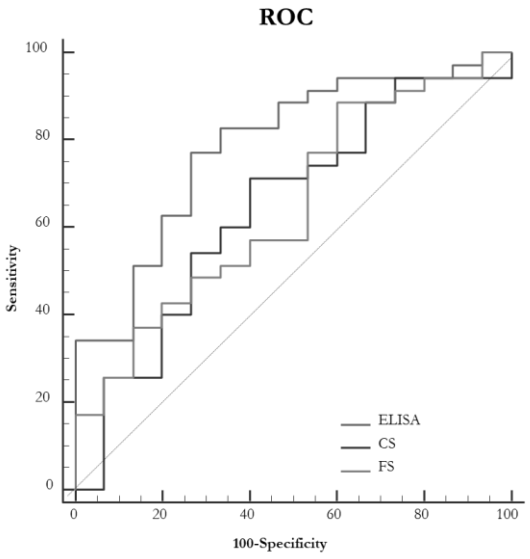
BSI	OR (95% CI)	p-value	AUC (95% CI)
ELISA	1.1 (1.0-1.1)	0.005	0.74 (0.60-0.89)
CS	1.0 (1.0-1.1)	0.34	0.62 (0.45-0.78)
FS	1.1 (1.0-1.1)	0.03	0.66 (0.51-0.81)

Pairwise comparison of ROC curves

ELISA ~ CS	
Difference between areas	0,126
95% Confidence Interval	0,0157 to 0,237
Significance level	P = 0,0252
ELISA ~ FS	
Difference between areas	0,0797
95% Confidence Interval	-0,0379 to 0,197
Significance level	P = 0,1840
CS ~ FS	
Difference between areas	0,0467
95% Confidence Interval	-0,0693 to 0,163
Significance level	P = 0,4300

BSI	Sensitivity, %	Specificity, %	PPV, %	NPV, %
ELISA	65.2	74.1	68.2	71.4
CS	34.8	74.1	53.3	57.1
FS	52.2	77.8	66.7	65.6

Figure B. Comparison of ROC curves of NE activity evaluated through different methods and sputum purulence in the bronchiectasis cohort



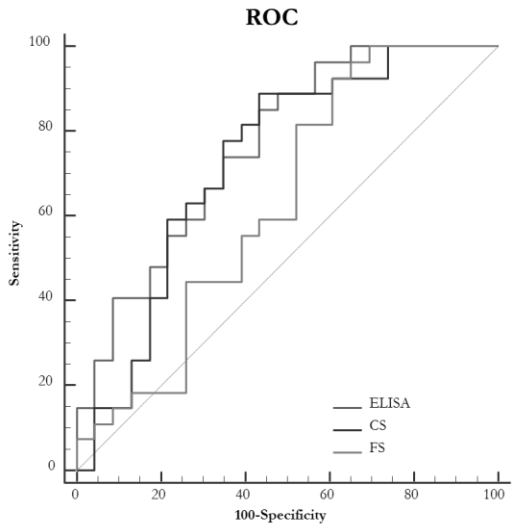
Mucopurulent/purulent sputum	OR (95% CI)	p-value	AUC (95% CI)
ELISA	1.1 (1.0-1.1)	0.004	0.79 (0.65-0.93)
CS	1.0 (1.0-1.1)	0.18	0.64 (0.47-0.82)
FS	1.1 (1.0-1.1)	0.09	0.65 (0.48-0.82)

Pairwise comparison of ROC curves

ELISA ~ CS	
Difference between areas	0.143
95% Confidence Interval	0.0327 to 0.253
Significance level	P = 0.0110
ELISA ~ FS	
Difference between areas	0.139
95% Confidence Interval	0.0331 to 0.245
Significance level	P = 0.0101
CS ~ FS	
Difference between areas	0.00381
95% Confidence Interval	-0.110 to 0.117
Significance level	P = 0.9475

Mucopurulent/purulent sputum	Sensitivity, %	Specificity, %	PPV, %	NPV, %
ELISA	82.9	60.0	82.9	60.0
CS	97.1	0.0	69.4	0.0
FS	100	0.0	70.0	-

Figure C. Comparison of ROC curves of NE activity measured through different methods in predicting any chronic infection in the bronchiectasis cohort



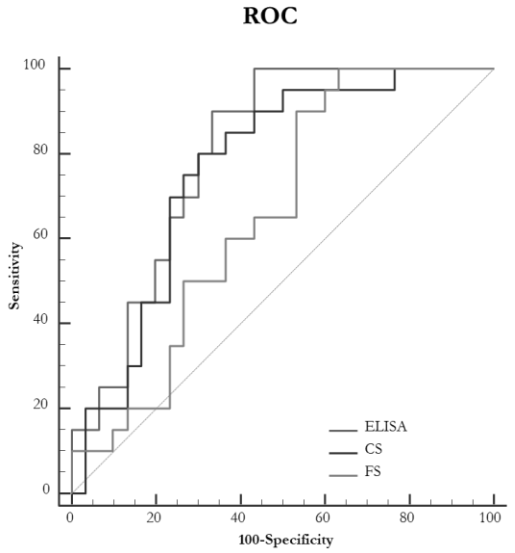
Chronic infection	OR (95% CI)	p-value	AUC (95% CI)
ELISA	1.1 (1.0-1.1)	0.004	0.76 (0.63-0.90)
CS	1.1 (1.0-1.1)	0.01	0.73 (0.58-0.88)
FS	1.0 (1.0-1.1)	0.23	0.63 (0.47-0.79)

Pairwise comparison of ROC curves

ELISA ~ CS	
Difference between areas	0.0338
95% Confidence Interval	-0.0772 to 0.145
Significance level	P = 0.5504
ELISA ~ FS	
Difference between areas	0.134
95% Confidence Interval	0.0161 to 0.251
Significance level	P = 0.0258
CS ~ FS	
Difference between areas	0.0998
95% Confidence Interval	-0.00843 to 0.208
Significance level	P = 0.0707

Chronic infection	Sensitivity, %	Specificity, %	PPV, %	NPV, %
ELISA	74.1	60.9	69.0	66.7
CS	70.4	65.2	70.4	65.2
FS	59.3	52.2	59.3	52.2

Figure D. Comparison of ROC curves of NE activity measured through different methods in predicting chronic *P. aeruginosa* infection in the bronchiectasis cohort



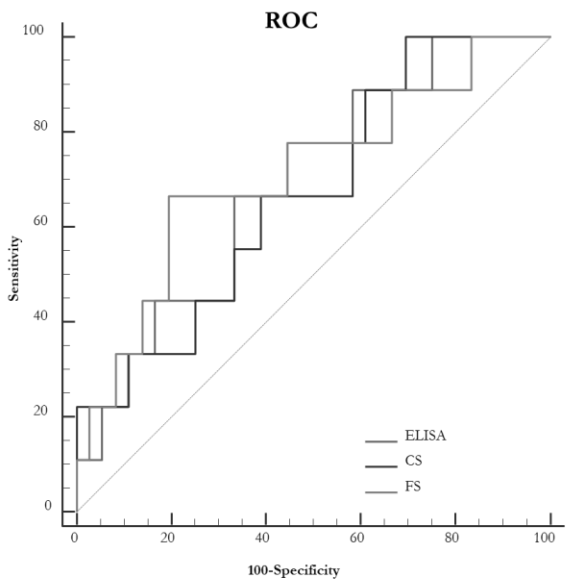
Chronic *P.aeruginosa* infection	OR (95% CI)	p-value	AUC (95% CI)
ELISA	1.1 (1.0-1.1)	0.001	0.80 (0.68-0.92)
CS	1.1(1.0-1.1)	0.006	0.77 (0.63-0.90)
FS	1.0 (1.0-1.1)	0.13	0.66 (0.51-0.81)

Pairwise comparison of ROC curves

ELISA ~ CS	
Difference between areas	0.0383
95% Confidence Interval	-0.0669 to 0.144
Significance level	P = 0.4752
ELISA ~ FS	
Difference between areas	0.143
95% Confidence Interval	0.0249 to 0.262
Significance level	P = 0.0177
CS ~ FS	
Difference between areas	0.105
95% Confidence Interval	-0.0159 to 0.226
Significance level	P = 0.0887

Chronic *P.aeruginosa* infection	Sensitivity, %	Specificity, %	PPV, %	NPV, %
ELISA	55.0	76.7	61.1	71.9
CS	50.0	76.7	58.8	69.7
FS	25.0	76.7	41.7	60.5

Figure E. Comparison of ROC curves of NE activity measured through three different methods and the presence of sputum quantity >50mL in the CF cohort



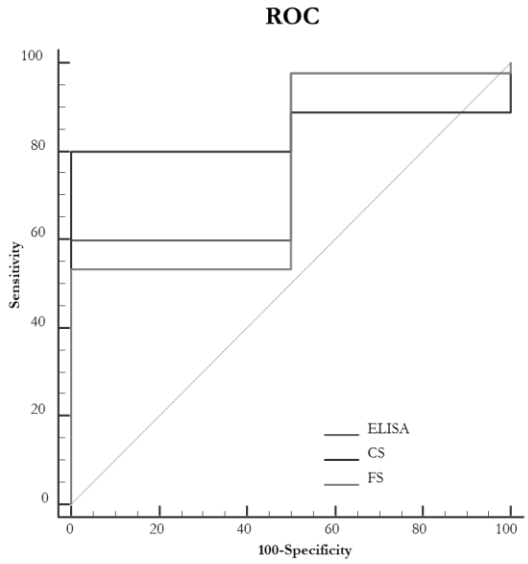
Sputum quantity >50 mL	OR (95% CI)	p-value	AUC (95% CI)
ELISA	1.0 (1.0-1.1)	0.09	0.69 (0.50-0.88)
CS	1.0 (1.0-1.1)	0.04	0.67 (0.47-0.87)
FS	1.1 (1.0-1.2)	0.04	0.71 (0.51-0.92)

Pairwise comparison of ROC curves

ELISA ~ CS	
Difference between areas	0.0216
95% Confidence Interval	-0.161 to 0.204
Significance level	P = 0.8164
ELISA ~ FS	
Difference between areas	0.0216
95% Confidence Interval	-0.0875 to 0.131
Significance level	P = 0.6979
CS ~ FS	
Difference between areas	0.0432
95% Confidence Interval	-0.153 to 0.240
Significance level	P = 0.6663

Sputum quantity >50 mL	Sensitivity, %	Specificity, %	PPV, %	NPV, %
ELISA	11.1	97.2	50.0	81.4
CS	22.2	97.2	66.7	83.3
FS	22.2	97.2	66.7	83.3

Figure F. Comparison of ROC curves of NE activity measured through three different methods and chronic infection with any bacteria in the CF cohort



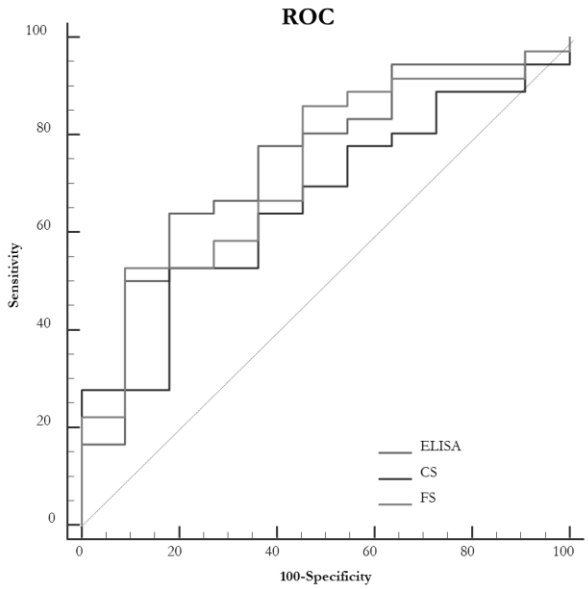
Chronic Infection	OR (95% CI)	p-value	AUC (95% CI)
ELISA	1.1 (1.0-1.1)	0.004	0.79 (0.65-0.89)
CS	1.0 (1.0-1.1)	0.01	0.84 (0.71-0.93)
FS	1.0 (1.0-1.1)	0.23	0.76 (0.61-0.87)

Pairwise comparison of ROC curves

ELISA ~ CS	
Difference between areas	0.0556
95% Confidence Interval	-0.409 to 0.520
Significance level	P = 0.8148
ELISA ~ FS	
Difference between areas	0.0333
95% Confidence Interval	-0.0537 to 0.120
Significance level	P = 0.4526
CS ~ FS	
Difference between areas	0.0889
95% Confidence Interval	-0.441 to 0.619
Significance level	P = 0.7425

Chronic Infection	Sensitivity, %	Specificity, %	PPV, %	NPV, %
ELISA	74.1	60.9	69.0	66.7
CS	70.4	65.2	70.4	65.2
FS	56.3	52.2	59.3	52.2

Figure G. Comparison of ROC curves of NE activity measured through three different methods and chronic infection with *P. aeruginosa* in the CF cohort



Chronic *P. aeruginosa* infection	OR (95% CI)	p-value	AUC (95% CI)
ELISA	1.1 (1.0-1.2)	0.04	0.75 (0.58-0.91)
CS	1.0 (1.0-1.1)	0.12	0.66 (0.49-0.83)
FS	1.2 (1.0-1.4)	0.07	0.73 (0.56-0.90)

Pairwise comparison of ROC curves

ELISA ~ CS	
Difference between areas	0.0859
95% Confidence Interval	-0.141 to 0.312
Significance level	P = 0.4573
ELISA ~ FS	
Difference between areas	0.0177
95% Confidence Interval	-0.0622 to 0.0976
Significance level	P = 0.6645
CS ~ FS	
Difference between areas	0.0682
95% Confidence Interval	-0.152 to 0.289
Significance level	P = 0.5447

Chronic *P. aeruginosa* infection	Sensitivity, %	Specificity, %	PPV, %	NPV, %
ELISA	100.0	0.0	76.6	-
CS	97.2	0.0	76.1	0.0
FS	100.0	0.0	76.6	-

Figure H. Correlation between NE activity measured through three different methods and FEV_1% in the CF cohort

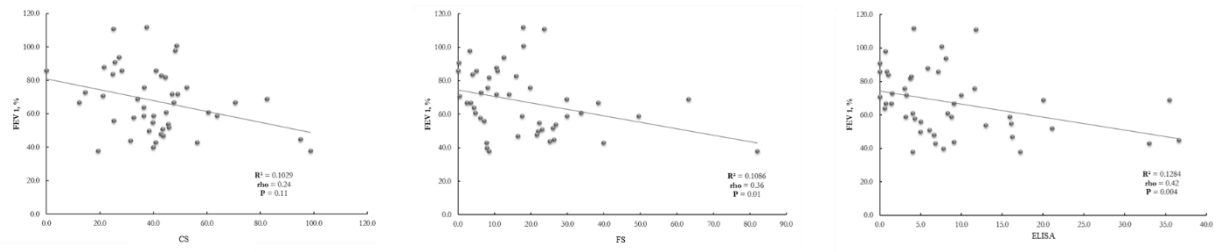
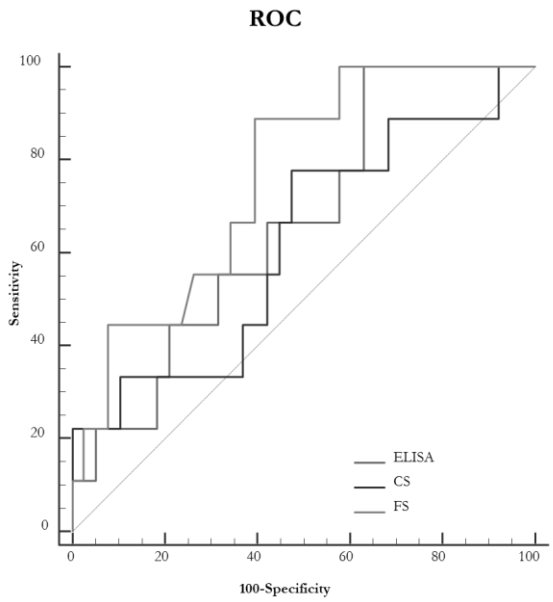


Figure I. Comparison of ROC curves of NE activity measured through three different methods and $FEV_1\%$ <50% evaluation in the CF cohort



FEV 1 <50%	OR (95% CI)	p-value	AUC (95% CI)
ELISA	1.0 (1.0-1.2)	0.12	0.66 (0.47-0.85)
CS	1.0 (1.0-1.1)	0.07	0.62 (0.40-0.84)
FS	1.1 (1.0-1.2)	0.03	0.76 (0.60-0.92)

Pairwise comparison of ROC curves

ELISA ~ CS	
Difference between areas	0.0439
95% Confidence Interval	-0.137 to 0.224
Significance level	P = 0.6337
ELISA ~ FS	
Difference between areas	0.0980
95% Confidence Interval	-0.0259 to 0.222
Significance level	P = 0.1210
CS ~ FS	
Difference between areas	0.142
95% Confidence Interval	-0.0121 to 0.296
Significance level	P = 0.0710

FEV 1 <50%	Sensitivity, %	Specificity, %	PPV, %	NPV, %
ELISA	11.1	100.0	100.0	82.6
CS	22.2	100.0	100.0	84.4
FS	22.2	97.4	66.7	84.1

Chapter 3

3 Patients' characteristics and sputum microbiome

3.1 Sputum microbiome composition and diversity in bronchiectasis

3.1.1 Introduction

As previously discussed in chapter 1 of this thesis, pulmonary microbial community is involved in the pathophysiology of bronchiectasis and in disease progression. Airway dysbiosis has been previously evaluated in patients with chronic respiratory diseases, and low microbiome diversity in sputum seems to be associated with disease severity and worse clinical outcomes in patients with CF, COPD and asthma [1–4].

Airway dysbiosis is also associated withdisease severity and neutrophilic inflammation in bronchiectasis [4]. Some aspects of the relationship among bronchiectasis aetiology, patients' characteristics and microbiome have been evaluated across previous experiences with preliminary data suggesting a link among microbiome inter patients diversity and bronchiectasis aetiology [5]. Furthermore, compositional studies identified *Proteobacteria*-dominated sputum microbiome to be associated to severer disease compared to *Firmicutes*-dominant patients[6, 7]. Finally, airways microbiome is also important in priming host immunity, and dysbiosis may have effects on host response to noxious triggers [8]. We hypothesize that specific clinical characteristics and aetiologies of bronchiectasis could be associated with low sputum microbiome diversity in this population[9, 10]. The aim of our study was to describe clinical characteristics associated with low microbiome diversity in sputum of adults with bronchiectasis.

3.1.2 Material and Methods

3.1.2.1 Study design and population

An observational, cross-sectional study was carried out at Bronchiectasis Program of the Respiratory Department, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, between March 2017 and March 2019. Consecutive adults (aged ≥18 years) with clinically (daily sputum production) and radiologically (≥1 lobe involvement on chest CT) confirmed bronchiectasis were recruited during clinical stability (≥1 month from the last exacerbation and antibiotic course). Patients with either CF or traction bronchiectasis due to pulmonary fibrosis or receiving long-term inhalation antibiotics were excluded. The study was approved by the ethical committee of the hospital (255_2020, Comitato Etico Milano Area 2), and all subjects provided written informed consent to participate.

3.1.2.2 Clinical variables

Demographics, comorbidities, disease severity, aetiology of bronchiectasis, respiratory symptoms, sputum evaluation, radiological and functional and biological characteristics in stable state were recorded, see online supplement Chapter "Sputum Neutrophil Elastase associates with microbiota and *P. aeruginosa* in bronchiectasis" of this thesis for further details. Modified BSI and E-FACED excluding microbiological evaluation were used in this section.

3.1.2.3 Microbiome evaluation

High-throughput sequencing of bacterial 16S rRNA-encoding gene amplicons (V3-V4 region) was performed according to previously published studies [11, 12]. A first aliquot of sputum plug was used for DNA extraction. DNA extraction followed a procedure previously developed in the laboratory, whereas the sequencing procedure used the 16S metagenomic sequencing library preparation protocol (Illumina, San Diego, CA, USA) [11, 12]. Reagent control samples and mock DNA were sequenced and analysed in parallel. Sequencing was carried out on the MiSeq (Illumina, San Diego, CA, USA) platform, using a 2_ 300 cycle V3 kit according to standard Illumina sequencing protocols as previously described in this thesis (Section "Sputum Neutrophil Elastase associates with microbiota and *P. aeruginosa* in bronchiectasis").

3.1.2.4 Inflammatory biomarkers evaluation

A second aliquot of mucous plug was used for the detection of inflammatory biomarkers. Sputum was diluted 8X in PBS, vortexed, and centrifuged at 4°C for 15 minutes at 3,000g. Supernatants were stored at -80°C. For the assessment of active neutrophil elastase (aNE) ProteaseTag® Active Neutrophil Elastase Immunoassay (Proaxis, Belfast, UK) was used as per manufacturer's instructions [13] . TNFα, IL6, IL10, and IL1β were assessed through commercial ELISA assays (Cusabio Technology LLC, Houston, USA).

3.1.2.5 Bioinformatic analysis

Bioinformatic analysis was carried out as previously described [4]. Quantitative Insights into Microbial Ecology (QIIME2) pipeline was used to analyze 16s sequences reads. Sequences underwent de-multiplexing and quality filtering with default parameters. After demultiplexing, sequences were matched through the DADA2 pipeline generating amplicon sequence variants (ASV) and a frequency table [14, 15]. Quality control step filtered out non-microbiota sequences with a 97% homology to a Greengenes database. A classifier with 99% homology to the V3-V4 region was trained with remaining sequences assigned with taxonomy based on 99% homology [16]. No OTU was removed from the analysis.

ASV frequency table was used to report relative abundance of each type of bacteria. Shannon diversity index, Pielou' Evenness and richness were utilized to evaluate alpha diversity (within sample diversity). The DESeq2 (v3.5, R Bioconductor) package was used to evaluate differences between groups of 16S rRNA gene sequencing data through Differential gene expression analysis based on the negative binomial distribution [17]. Data were analysed at both genus and phylum levels.

3.1.2.6 Study groups

We a priori decided to split our population based on Shannon diversity median value in order to have two equally distributed groups. Patients with Shannon values <median level are included in the "low" group, while ones with Shannon entropy ≥ median level are reported as "high" group.

3.1.2.7 Statistical analysis

Variables were collected in an ad hoc electronic form. Qualitative variables were summarized with absolute and relative (percentage) frequencies, whereas quantitative variables with medians (interquartile ranges, IQR). A univariate and multivariate logistic regression analysis was carried out to assess the relationship between microbial diversity and clinical variables based on beta coefficients and related 95% confidence intervals.

The statistical software R version 4.0.1 (R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.) was used for all statistical computations.

3.1.3 Results

178 patients [46 (25.8%) males, age: 63 (50-71)] were enrolled in the study period. Patients enrolled showed a median (IQR) BSI of 5 (3-7), 2 (1-3) exacerbations in the previous year and an idiopathic aetiology in the 61.8% of patients. Full descriptive analysis is reported in table 1.

Table 1. Descriptive analysis.

Variables	n= 178
Low diversity Shannon, n (%)	89/178 (50.0)
Demographics	
Median (IQR) age, years	63 (50-71)
Age >65 years, n (%)	71/178 (39.9)
Age >75 years, n (%)	22/178 (12.4)
Males, n (%)	46/178 (25.8)
Median (IQR) BMI, kg/m^2	21.6 (19.0-24.8)
Underweight (BMI <18.5 kg/m^2), n (%)	30/177 (17.0)
Former or current smoker, n (%)	75/178 (42.1)
Disease severity	
Median (IQR) Modified BSI	5 (3-7)
Median (IQR) Modified E-FACED	2 (1-4)
Radiology	
Median (IQR) Reiff score	4 (3-8)
Mean (SD) no. of lobes	4 (1.6)
4-6 lobes involved, n (%)	101/177 (47.1)
Aetiology	
Idiopathic aetiology, n (%)	110/178 (61.8)
PCD aetiology, n (%)	20/178 (11.2)
Immunodeficiency aetiology, n (%)	18/178 (10.1)
Post-infective aetiology, n (%)	14/178 (7.9)
Other aetiology, n (%)	16/178 (9.0)
Clinical status and diagnostic results	
Median (IQR) exacerbation previous year	2 (1-3)
≥2 exacerbations previous year, n (%)	103/177 (58.2)
≥3 exacerbations previous year, n (%)	55/177 (31.1)
Median (IQR) hospitalization previous year	0 (0-0)
≥1 hospitalization previous year, n (%)	26/177 (14.7)
Emphysema, n (%)	22/178 (12.4)
Median (IQR) Vitamin D	23.5 (14.1-32.0)
Relevant comorbidities	
Median (IQR) BACI	0 (0-3)
COPD, n (%)	18/178 (10.1)
GERD, n (%)	75/178 (42.1)
Pulmonary function	
Mean (SD) FEV_1, %	79.3 (23.8)
FEV_1 ≤35, n (%)	8/176 (4.6)
FEV_1 ≤50, n (%)	19/176 (10.8)
Therapy	
PPI, n (%)	60/178 (33.7)
LABA, n (%)	99/178 (55.6)
LAMA, n (%)	76/178 (42.7)
ICS, n (%)	64/178 (36.0)

Azithromycin, n (%)	12/178 (6.7)

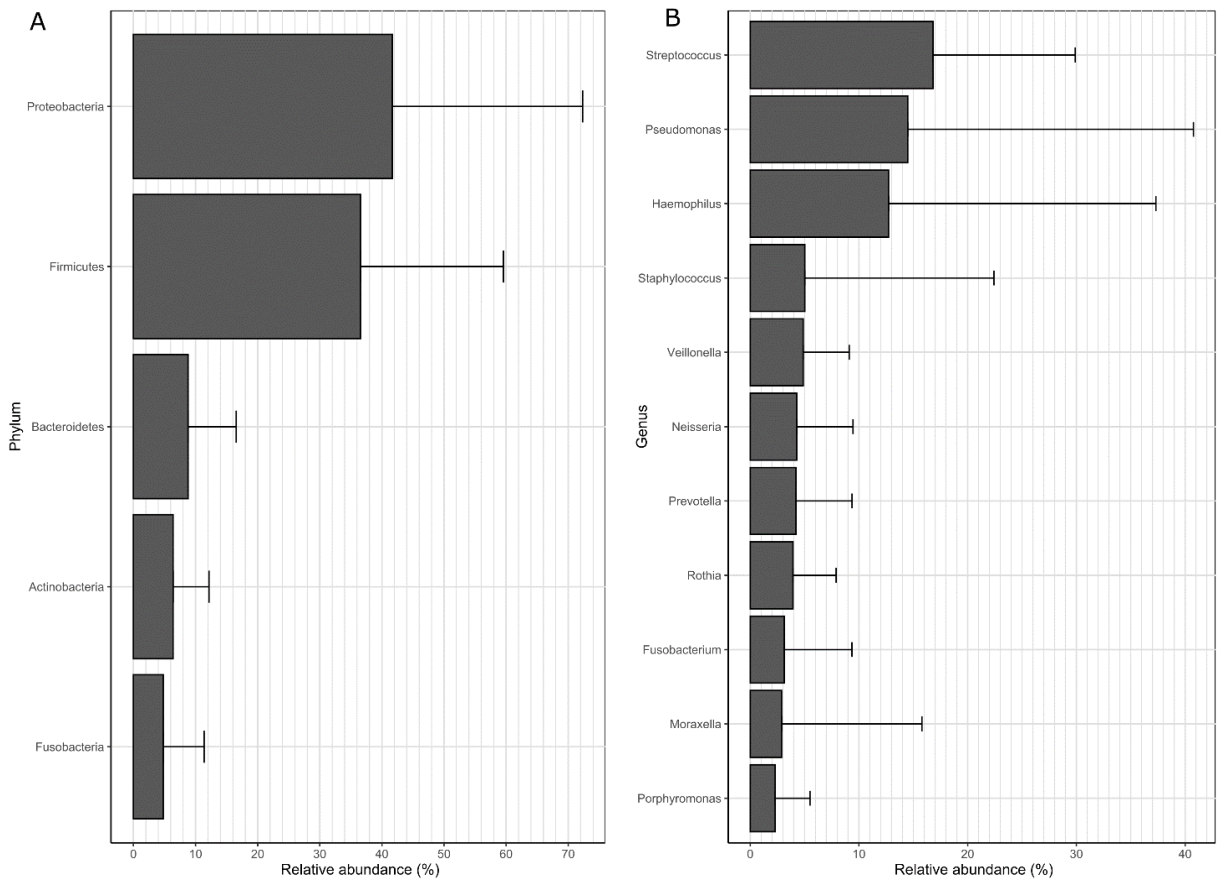
3.1.3.1 Microbiome

Microbiome analysis was performed in all 178 patients and positive and negative controls. 210 sequences were found in negative control, filtered out after quality control step.

Compositional analysis was performed at both phylum and genus level. The most abundant phyla (>2%) identified in our samples were:*Proteobacteria* (41.7%), *Firmicutes* (36.6%), *Bacteroidetes* (8.8%), *Fusobacteria* (4.8%) and *Actinobacteria* (6.4%), see figure 1A.

At genus level, most aboundant bacteria reported were *Streptococcus* (16.8%), *Pseudomonas* (14.5%), *Haemophilus* (12.7%), *Staphylococcus* (5%), *Veillonella* (4.8%), *Neisseria* (4.3%), *Prevotella* (4.3%), *Rothia* (3.9%), *Fusobacterium* (3.1%), *Moraxella* (2.9%) and *Porphyromonas* (2.3%), see figure 1B.

Figure 1. Relative abundance of phyla (A) and genera (B) of the study population



Median (IQR) within-sample diversity was reported as Shannon diversity 4.4 (3.1-5.6), Pielou' evenness 0.8 (0.6-0.9), and richness 61 (39-87).

3.1.3.2 Study groups

Patients were divided in the two study groups based on median Shannon diversity levels. Specifically, 89 patients were enrolled in each group.

Patients belonging to the microbiome Low-diversity group had a severer disease, as showed by the median (IQR) modified BSI [low 5 (3.0-6.3) VS. high 5 (3-9); p-value= 0.03] and the median (IQR) modified E-FACED [low 2 (1-3) VS. high 3 (1-4); p-value= 0.02], higher radiological severity measured by the Reiff score and by the number of involved pulmonary lobes [median (IQR) Reiff score: low 4 (4-4) VS. high 4 (2-6); p-value= 0.002; median (IQR) number of lobes low 4 (3-6) VS. high 3 (2-4); p-value= 0.004] (Table 1). Furthermore, the prevalence of patients with primary ciliary dyskinesia (PCD) was higher in the Low-diversity group [low 15 (16.9 %) VS. high 5 (5.6%); p-value= 0.02], as well as in those with median (IQR) FEV_1<50%predict. [low 16 (18.2%) VS. high 3 (3.4%); p-value= 0.003].

Table 2. Comparison of clinical characteristics between low- and high-diversity groups.

Variables	Microbiome Low-diversity group (Shannon <4.4)	Microbiome High-diversity group (Shannon ≥4.4)	p-value
	n=89	n=89	
Demographics			
Median (IQR) age, years	62 (53.0-71.0)	64 (46.5-72.5)	0.59
Females, n (%)	62 (69.7)	70 (78.7)	0.17
Median (IQR) BMI, kg/m^2	21.9 (19-25)	21(19.1-24.4)	0.45
Underweight (BMI <18.5 kg/m^2), n (%)	16 (18.2)	14 (15.7)	0.66
Former or current smoker, n (%)	36 (40.5)	39 (43.8)	0.65
Disease severity			
Median (IQR) adjusted BSI (w/o microbiology)	5 (3-9)	5 (3-6.3)	0.03
Median (IQR) adjusted E-FACED (w/o microbiology)	3 (1-4)	2 (1-3)	0.02
Radiology			
Median (IQR) Reiff score	4 (4-4)	4 (2-6)	0.002
Mean (SD) No. of lobes	4 (3-6)	3 (2-4)	0.004
4+ lobes involvement, n (%)	61(68.5)	40(45.5)	0.002
Aetiology			
Idiopathic, n (%)	51 (57.3)	59 (66.3)	0.22
PCD, n (%)	15 (16.9)	5 (5.6)	0.02
Immunodeficiency, n (%)	10 (11.2)	8 (9.0)	0.62
Post-infective, n (%)	5 (5.6)	9 (10.1)	0.27
Other aetiologies, n (%)	8 (9.0)	8 (9.0)	1.00
Clinical status and diagnostic results			
Median (IQR) exacerbation previous year	2 (1-3)	2 (1-3)	0.93
≥2 exacerbations previous year, n (%)	51 (57.3)	52 (59.1)	0.81
≥3 exacerbations previous year, n (%)	25 (28.1)	30 (34.1)	0.39
≥1 hospitalization previous year, n (%)	17 (19.1)	9 (10.2)	0.10
Emphysema, n (%)	10 (11.2)	12 (13.5)	0.65
Median (IQR) Vitamin D, ng/mL	22.3 (14-32)	23.8 (16-31.8)	0.95

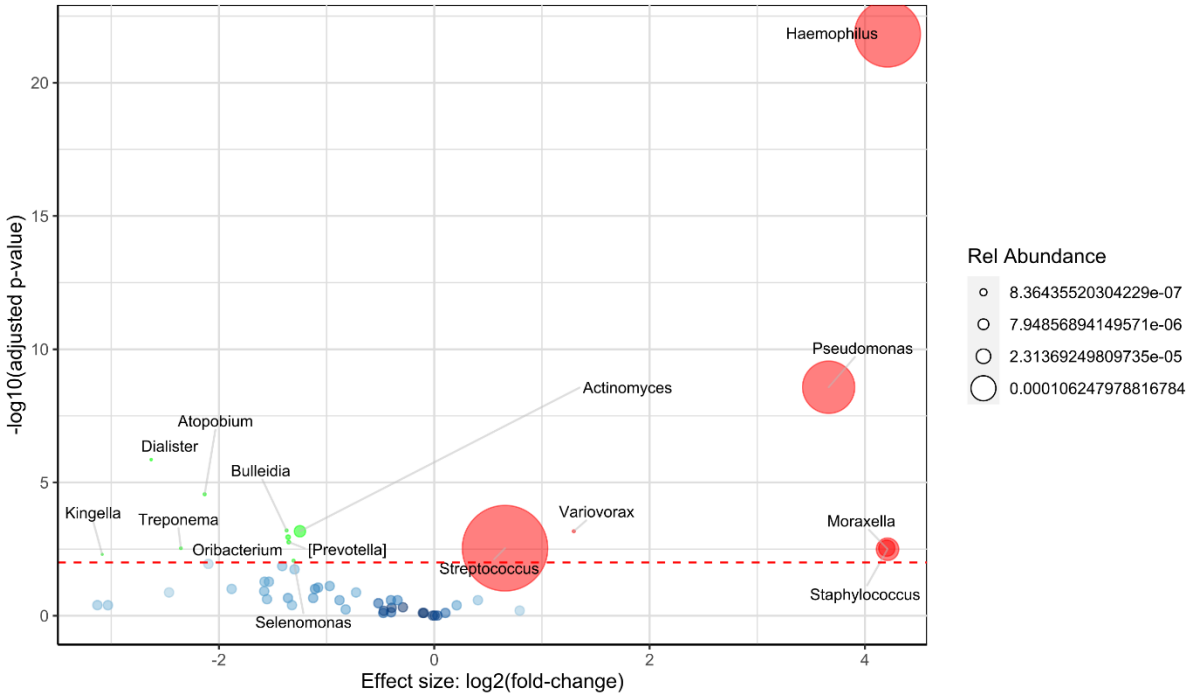
Quality of life			
Median (IQR) QoL-B questionnaire - Physical	46.7 (13.3-86.7)	63.4 (33.3-73.3)	0.23
Median (IQR) QoL-B questionnaire - Role	50 (33.3-80)	63.4 (53.3-80)	0.48
Median (IQR) QoL-B questionnaire - Vitality	55.6 (44.4-66.7)	44.4 (33.3-55.6)	0.39
Median (IQR) QoL-B questionnaire - Emotion	75 (50-91.7)	70.9 (41.7-83.3)	0.57
Median (IQR) QoL-B questionnaire - Social	66.7 (33.3-83.3)	62.5 (41.7-75.0)	0.58
Median (IQR) QoL-B questionnaire – Treatment Burden	66.7 (55.6-77.8)	66.7 (55.6-88.9)	0.51
Median (IQR) QoL-B questionnaire - Health	41.7 (16.7-58.3)	37 (25-50)	0.927
Median (IQR) QoL-B questionnaire - Respiration	70.4 (48.1-77.8)	72.3 (55.6-77.8)	0.53
Relevant comorbidities			
Median (IQR) BACI	0 (0-3)	0 (0-3)	0.28
COPD, n (%)	10 (11.2)	8 (9.0)	0.62
GERD, n (%)	39 (43.8)	36 (40.5)	0.65
Pulmonary function			
Mean (SD) FEV_1, %	77.0 (60.5-93.5)	82.5 (68.0-95.0)	0.13
$FEV_1 \leq 35$, n (%)	2 (2.3)	6 (6.8)	0.28
$FEV_1 \leq 50$, n (%)	16 (18.2)	3 (3.4)	0.003
Chronic therapy			
PPI, n (%)	32 (36.0)	28 (31.5)	0.53
LABA, n (%)	54 (60.7)	45 (50.6)	0.18
LAMA, n (%)	40 (44.9)	36 (40.5)	0.54
ICS, n (%)	30 (33.7)	34 (38.2)	0.64
Azithromycin, n (%)	8 (9.3)	4 (4.7)	0.37
Sputum inflammatory biomarkers			
Median (IQR) Active neutrophil elastase, µg/ml	23.2 (16.1-34.3)	15.6 (5.2-31.6)	0.0001
Median (IQR) TNFα, pg/mL	1019.4 (732.3-1485.8)	702.6 (270.6-1158.4)	0.01
Median (IQR) IL6, pg/mL	177.9 (119.1-286.4)	147.5 (27.8-238.0)	0.04
Median (IQR) IL1β, pg/mL	364935.1 (234603-607736.9)	326433.6 (187109.6-444182.2)	0.16
Median (IQR) IL10, pg/mL	31411.7 (16861.9-64290.2)	22284.4 (8551.5-34587.2)	0.004

BMI=Body Mass Index, BSI=Bronchiectasis Severity Index, PCD=Primary Ciliary Dyskinesia, COPD=Chronic obstructive pulmonary disease, GERD=Gastro-Esophageal Reflux Disease, PPI=Proton Pump Inhibitors, LABA=Long-acting β2-agonists, LAMA=Long-acting muscarinic antagonists, ICS=inhaled corticosteroids

3.1.3.3 Microbiome according to the study groups

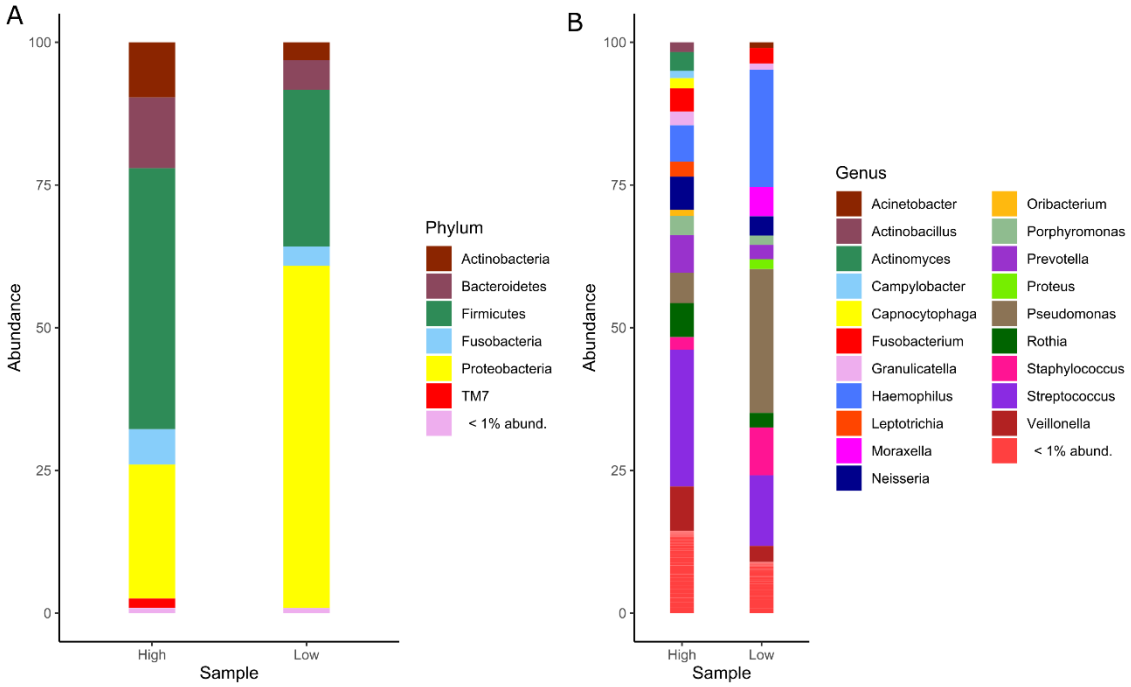
Differences were identified in the microbiome among the two study groups. DESeq2 analysis lead to the identification of several genera differentially enriched in the two groups. Mostly, *Pseudomonas, Haemophilus* and *Staphylococcus* are enriched in the low diversity group compare to the other, see figure 2 for complete enrichment evaluation.

Figure 2. Volcano plot showing a differential enrichment in the two study groups, red= increased in the low diversity group, green=decreased in the low diversity group.



Full description of the genera and phyla of the two study groups is reported in Figure 3.

Figure 3. Taxa barplots of phyla and genera according to the two study groups.



3.1.3.4 Microbiome diversity and inflammation

An increased median (IQR) levels of aNE [low 23.2 (16.1-34.3) µg/mL VS. high 15.6 (5.2-31.6) µg/mL; p-value= 0.0001], IL-6 [low 177.9 (119.1-286.4) pg/mL VS. high 147.5 (27.8-238.0) pg/mL; p-value= 0.04], and IL-10 [low 31,411.7 (16,861.9-64,290.2) pg/mL VS. high- 22,284.4 (8,551.5-34,587.2) pg/mL; p-value= 0.004] were found in the sputum of Low-diversity patients (Table 2).

3.1.3.5 Shannon entropy and clinical characteristics – Univariate analysis

Logistic regression identified an increased odd ratio (OR) for disease severity in terms of both modified BSI and modified E-FACED respectively OR (95% CI) 1.1 (1.0-1.2), p=0.01 and 1.3 (1.1-1.6), p=0.008 in predicting low Shannon diversity. Moreover, the number of lobes involved was identified as a candidate predictor of low sputum diversity with an OR of 2.6 (1.4-4.8), p=0.002 when 4-6 lobes are involved. PCD aetiology seems to be involved in low sputum diversity prediction with an OR of 3.4 (1.2-9.8), p=0.02. Finally, FEV_1%>50 was reported with 6.3 (1.8-22.5) OR, p=0.005. The complete univariate analysis is reported in Table 3.

Table 3. Univariate analysis to assess the relationship between demographic, epidemiological, clinical characteristics, and low diversity Shannon.

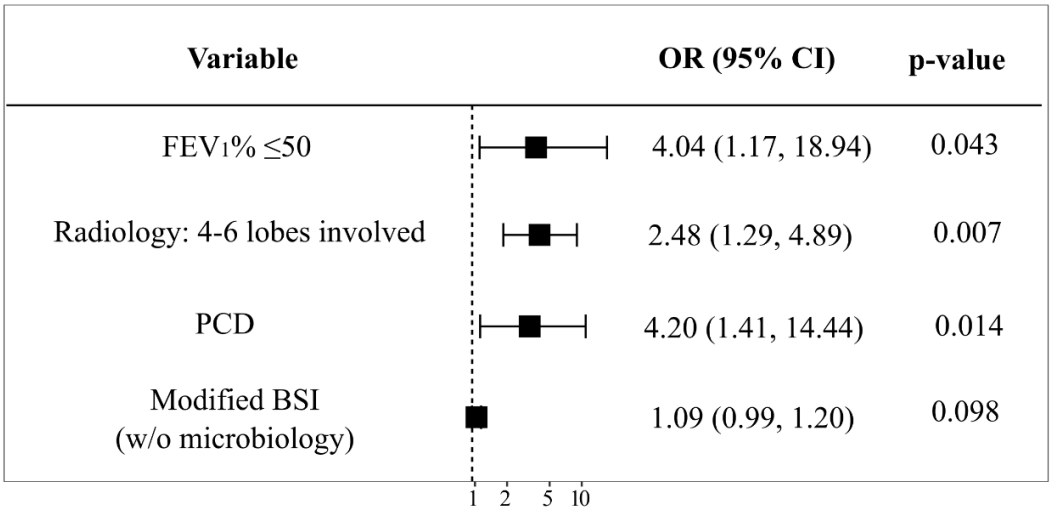
Variables	OR (95% CI)	p-value
Demographics		
Median (IQR) age, years	1.0 (1.0-1.0)	0.88
Age >65 years	1.7 (0.9-3.1)	0.09
Age >75 years	1.5 (0.6-3.8)	0.37
Males	1.6 (0.8-3.2)	0.17
BMI, kg/m^2	1.0 (0.9-1.0)	0.54
Underweight (BMI <18.5 kg/m^2)	1.2 (0.5-2.6)	0.66
Former or current smoker	0.9 (0.5-1.6)	0.65
Disease severity		
Modified BSI (without microbiology)	1.1 (1.0-1.2)	0.01
Modified E-FACED (without microbiology)	1.3 (1.1-1.6)	0.008
Radiology		
Reiff score	1.0 (1.0-1.2)	0.01
No. of lobes	1.3 (1.1-1.6)	0.007
4-6 lobes involvement	2.6 (1.4-4.8)	0.002
Aetiology		
Idiopathic aetiology	0.7 (0.4-1.3)	0.22
PCD aetiology	3.4 (1.2-9.8)	0.02
Immunodeficiency aetiology	1.3 (0.5-3.4)	0.62
Post-infective aetiology	0.5 (0.2-1.6)	0.27
Other aetiology	1.0 (0.4-2.8)	1
Clinical status		
Exacerbation previous year	1.0 (0.8-1.1)	0.59
≥2 exacerbations previous year	0.9 (0.5-1.7)	0.81
≥3 exacerbations previous year	0.8 (0.4-1.4)	0.39
hospitalization previous year	1.4 (0.8-2.4)	0.29
≥1 hospitalization previous year	2.1 (0.9-4.9)	1
Emphysema	0.8 (0.3-2.0)	0.65
Vitamin D	1.0 (1.0-1.0)	0.45

Relevant comorbidities		
BACI	1.1 (0.9-1.3)	0.23
COPD	1.3 (0.5-3.4)	0.62
GERD	1.2 (0.6-2.1)	0.65
Pulmonary function		
Mean (SD) FEV_1, %	1.0 (1.0-1.0)	0.07
FEV_1 % ≤35	3.1 (0.6-16.0)	0.17
FEV_1 % ≤50	6.3 (1.8-22.5)	0.005
Therapy		
PPI	1.2 (0.7-2.3)	0.53
LABA	1.5 (0.8-2.7)	0.18
LAMA	1.2 (0.7-2.2)	0.55
ICS	0.8 (0.5-1.5)	0.53
Azithromycin	2.1 (0.6-8.0)	0.25

3.1.3.6 Shannon entropy and clinical characteristics – Multivariate analysis

Multivariate analysis performed considering significant variables from univariate analysis (FEV_1%<50, 4-6 lobes involved, PCD and disease severity –BSI) resulted in the identification of FEV_1% <50, radiology and PCD as contributing factors to low microbiota diversity in sputum with respectively OR (95% CI) 4.04 (1.17, 18.94), p=0.043, 2.48 (1.29,4.89), p=0.007 and 4.20 (1.41, 14.44), p=0.014. The complete multivariate analysis is reported in figure 2.

Figure 2: Multivariate analysis for low Shannon diversity.



3.1.4 Discussion

Our study on microbiome diversity in a population of adults with bronchiectasis showed that FEV_1<50%predict., a radiological involvement of at least 4 lobes on HRCT scan, and the diagnosis of PCD are independently associated with a low microbiome diversity in sputum. Furthermore, a specific enrichment in *Pseudomonas, Haemophilus* and *Staphylococcu*s, as well as airway inflammation, characterises the sputum microbiome of bronchiectasis patients with low diversity.

Firstly, our data demonstrated a clear association between low respiratory function (FEV_1<50%predict) with low microbiome diversity in sputum. This association has been studied in chronic respiratory diseases including CF and bronchiectasis. Studies in CF reported a direct correlation between FEV_1 and Shannon diversity, whereas experiences in bronchiectasis associated respiratory function decline with low microbiome diversity [2, 18].

Secondly, the relationship between airways microbiome and radiological impairment offers new evidence pointing out the interplay of tissue damage, infection, and inflammation in the pathophysiology of bronchiectasis [19, 20]. An association with neither microbiome composition nor diversity has been evaluated so far in bronchiectasis. Engel *et. al.* speculated that radiological differences may be involved in a modification in the interaction among bacterial community and host response leading to an adjustment of microbial community to radiology [20].

Thirdly, among the different aetiologies evaluated, only PCD was independently associated with low sputum microbiome diversity [21]. Rogers and colleagues described microbiome composition of sputum of a mixed population of 24 children and adults with PCD enrolled in stable state and found a negative correlation between *P. aeruginosa* abundance and FEV_1%predict[22]. No relationship between clinical characteristics and microbiome diversity was found in this study [22]. Our data expand current knowledge identifying the PCD population as the most affected in terms of microbiome diversity among bronchiectasis patients and underlines the importance of excluding this genetic disorder when evaluating the aetiology of bronchiectasis. Further translational studies are needed to better define the mechanisms supporting the association between PCD and low microbiome diversity [22].

The association we found between FEV_1<50%predict., a radiological involvement of at least 4 lobes on HRCT scan, and the diagnosis of PCD with a low microbiome diversity in sputum do not allow us to unravel cause-effect relationship. We can speculate that there might be a two-way relationship among these effectors. From one side, patient characteristics might impact

pulmonary physiology and ecosystem as mucus volume, oxygen tension, and pH [23, 24]. From the other side, the airway dysbiosis may also have an impact on host immunity and, subsequently, on patient conditions [20, 25]. Specific interventions focusing on both microbiome and clinical characteristics may help clinicians to directly intervene on the pathophysiological vicious cycle in bronchiectasis.

In terms of exacerbations, previous studies identified an association between their frequency and a decreased microbiome diversity in bronchiectasis [9, 26]. These findings are not confirmed in the present study. We can speculate that geographical heterogeneity, as well as different severity distribution of patients, may influence exacerbation rates and their relationship with microbiome. Although the cause of bronchiectasis exacerbations is still matter of debate, it might be associated with the acquisition of a new pathogen [26]. The ability of a single pathogen to antagonize the existing microbiome and to cause exacerbations is still under evaluation and a focus on the frequent exacerbator profile and microbiome diversity might unravel this association.

In terms of microbiota composition, our findings are in line with previously published data [6]. *Proteobacteria* and *Firmicutes* are the most prevalent phyla in bronchiectasis and *Proteobacteria* are the dominant phylum in the low-diversity group. High levels of *Proteobacteria* were previously associated to dysbiosis and *P. aeruginosa* predominance in bronchiectasis [7]. In addition, *Pseudomonas* (*Proteobacteria*), *Haemophilus*(*Proteobacteria*) and *Staphylococcus* (*Firmicutes)* emerged to be enriched in the low-diversity group [27]. These bacteria are known pathogenic colonizers of bronchiectasis airways. The dominance of pathogenic genera was already associated to dysbiosis and poor patients' conditions in bronchiectasis and we may speculate that the high presence of these genera may be responsible for an unbalance in airway microbiome, leading to a further contribution of the microbiological component to the pathophysiology vicious vortex associated with bronchiectasis. We can speculate that the increase of these genera may be due to inter-genera interaction of these bacteria that may lead to the predominance/shift in composition of sputum microbiome[7]. However, data on *Streptococcus* are contradictory. While it seems to be enriched in the low diversity group from DESeq analysis, the log fold change (0.66) is low and the descriptive analysis shows a decrease in relative abundance in the Low-diversity group. Bioinformatic methods are heterogeneous in the definition of a threshold for log fold change at DESeq analysis that may be omitted or set between 1 and 2.5. For this reason, we decided not to set it in order to avoid loss of important

information. However, this choice may lead to contradictory results and further studies may be needed to clarify this specific point.

In terms of airway inflammation, our findings confirmed previous experiences showing a link between airway inflammation and low microbiome diversity with high levels of aNE, IL-6, and IL-10 detected in our patients in the Low-diversity group [4].

Different limitations of our study should be acknowledged. 1) Its cross-sectional design did not allow us the evaluate microbiome dynamics; 2) The mono-centre enrolment of Italian patients hinders the assessment of the geographical heterogeneity on microbiological variability; 3) The study included bronchiectasis patients who could provide sputum samples. This choice excluded an interesting group of patients who may be recruited in future studies analysing microbiome from deep-lung samples; 4) Our choice of sputum as matrix for microbiome analysis may limit our study conclusions. Deep-lung samples, such as BAL or BAS, could avoid oral microbiome contamination. However, those specimens are collected with invasive procedures, reducing their potential point-of-care adoption.5) We did not consider modifications in microbiome due to long term antibiotic treatment and the number of patients in azithromycin therapy are too low to be compared. Further studies investigating the role of inhaled antibiotics and long terms macrolide may lead to a better understanding of the role of these therapy on microbiome.6) Finally, our analysis has been focused only on bacteria, while other microorganisms might act as confounders of our findings.

This is the first study evaluating the association between airway dysbiosis, patients' clinical conditions, and bronchiectasis aetiologies through a multivariable approach. We also described both bacterial community and inflammatory status in cases of low microbiome diversity, giving a detailed perspective on the lung compartment.

In conclusion, FEV_1<50%predict., a radiological involvement of at least 4 lobes, and the diagnosis of PCD are the only independent factors associated with low microbial diversity in sputum of bronchiectasis patients. Interventions aimed at modifying airway dysbiosis might help stabilize or address the issue of the functional and radiological progression in patients with bronchiectasis, especially in those affected by PCD.

3.1.5 References

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3.2 Bronchiectasis aetiologies and sputum microbiome

The following paragraphs represent preliminary studies arising from a secondary analysis of the above presented data. These preliminary analyses represent the first step to design future studies.

3.2.1 Primary Ciliary Dyskinesia

3.2.1.1 Introduction

Among aetiologies, Primary Ciliary Dyskinesia (PCD) resulted to be associated to low microbiome diversity in chapter 4.1 of this thesis. Data in literature on PCD patients (both children and adults) reported a negative correlation between *P. aeruginosa* abundance and FEV_1%predict [1]. Moreover, a recent study reported clustering in Principal Coordinates Analysis (PCoA) of inter-sample diversity in PCD aetiology. The study included patients with idiopathic bronchiectasis, ABPA, immunodeficiency, PCD, post-infective and others. Although some data on PCD microbiome are present in literature, no specific characterization of the microbiome and inflammatory status of lung was made for these patients. The aim of this study was to describe sputum microbiome, along with airway inflammation, in patients with PCD compared to a group of idiopathic and post infective.

3.2.1.2 Materials and methods

3.2.1.2.1 Study design and population

A secondary analysis of the biobank and the clinical database of the Bronchiectasis Program of the Respiratory Department, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, was conducted. Adults (≥18 years) referring to Bronchiectasis Program between March 2017 and March 2019 and with either primary or secondary immunodeficiency or idiopathic aetiology were included in the study.

Patients with PCD were matched with idiopathic or post infective for age (+/-5 years), sex and FEV_1%predicted< 50%.

3.2.1.2.2 Study procedures

Clinical data, along with microbiome and neutrophilic inflammation ones were analysed for the PCD and idiopathic or post infective groups and reported according to methods previously described in this thesis (Section "Sputum Neutrophil Elastase associates with microbiota and *P. aeruginosa* in bronchiectasis").

3.2.1.3 Results

20 patients were enrolled for each group of this preliminary study [Male 11 (55%); age PCD 43.5 (31.8-58.5), idiopathic/post infective 43.5 (31.8-60.0); FEV_1%predicted PCD 76.5 (61.0-94.0), Idiopathic/Post infective 82 (56.5-90.0)].

Clinical characteristic resulted to be similar across the two groups in terms of all the considered variables. Specifically, disease severity [BSI PCD 5.5 (3.5-10.0) vs idiopathic/post infective 4.0 (3.0-6.5), p=0.21], number of exacerbations in the previous year [PCD 2.0 (1.0-2.5) vs idiopathic/post infective 1.5(1.0-3.5), p=0.68] and number of patients with 1+ hospitalization in the previous year [PCD 1 (5.0) vs idiopathic/post infective 2 (10.0), p=1.00] were similar in the two study groups (Table 1).

Table 1. Description of the study population according to the study groups.

Variable	PCD	Idiopathic/Post infective	p-value
Sex (male), n (%)	11 (55.0)	11 (55.0)	1.00
Median (IQR) age, years	43.5 (31.8-58.5)	43.5 (31.8-60.0)	0.86
Former or current smoker, n (%)	6 (30.0)	9 (45.0)	0.33
Median (IQR) FEV_1%predicted	76.5 (61.0-94.0)	82 (56.5-90.0)	0.92
Median (IQR) Bronchiectasis severity index	5.5 (3.5-10.0)	4.0 (3.0-6.5)	0.21
Median (IQR) Exacerbations in the previous year	2.0 (1.0-2.5)	1.5(1.0-3.5)	0.68
≥ 1 hospitalization in the previous year, n (%)	1 (5)	2 (10)	1.00
Inhaled antibiotics, n (%)	0 (0.0)	2 (10.0)	0.49
Azithromycin, n (%)	2 (10.0)	3 (15.0)	1.00

3.2.1.3.1 Microbiome analysis

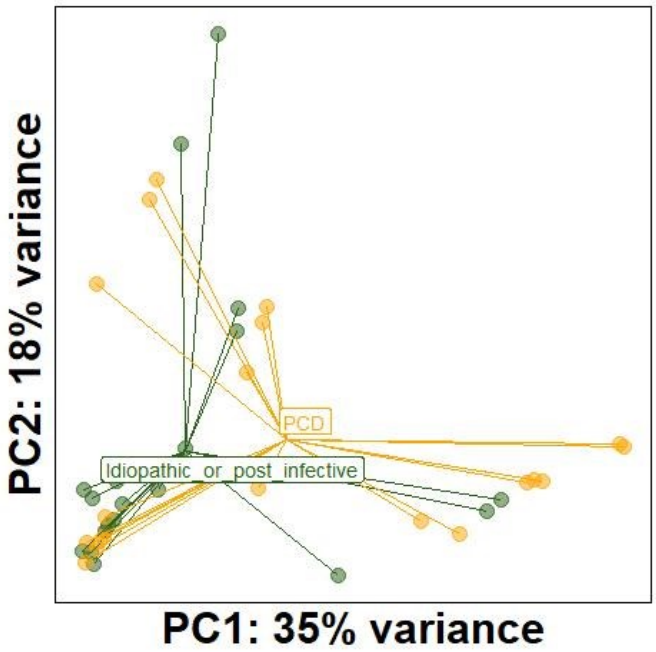
Alpha diversity analysis reported no significant difference of Shannon, Evenness and Richness dominance in the PCD group compared with the Idiopathic/Post infective one (Table 2), although the three within-patients metrics resulted to be decreased in the PCD group compared to the Idiopathic/Post infective one.

Table 2. Comparison of alpha diversity matrices according to the study groups

Variable	PCD	Idiopathic/Post infective	p-value
Median (IQR) Shannon	3.8 (2.1-5.2)	4.81 (2.86-5.92)	0.17
Median (IQR) Richness	36.5 (17.8-81.8)	60.5 (39.5-102.0)	0.10
Median (IQR) Evenness	0.7 (0.5-0.8)	0.8 (0.5-0.9)	0.23

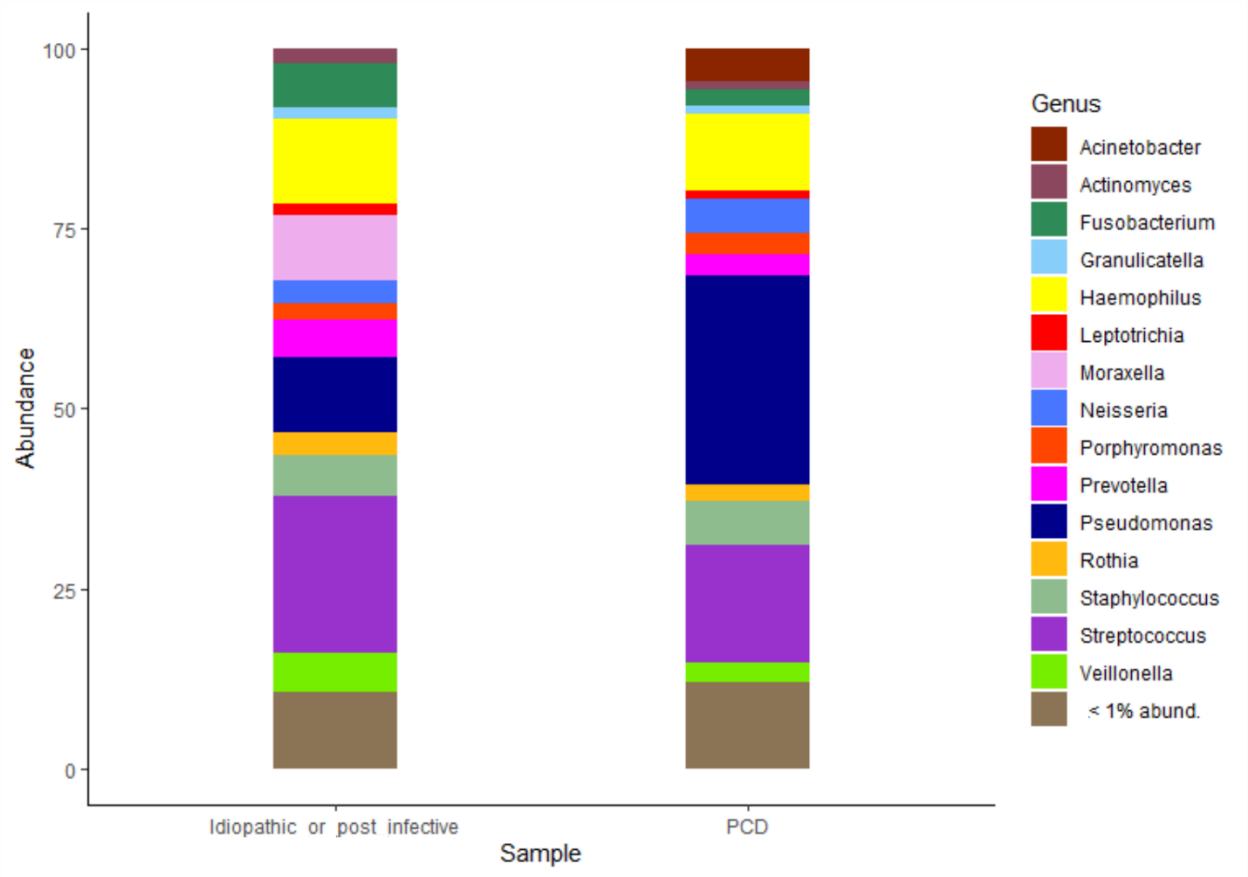
Similar results emerged from beta diversity analysis through Bray-Curtis dissimilarity. PERMANOVA analysis did not detect any difference between groups, p=0.15 (Figure 1)

Figure 1. PCoA based on Bray Curtis dissimilarity according to the two study groups



No statistical enrichment was detected through DESeq2 analysis. Genera barplots describing the two groups are reported in Figure 2.

Figure 2. Genera barplots according to the two study groups



3.2.1.3.2 Molecular detection of respiratory pathogens

Real time analysis highlighted a statistical increase of *P. aeruginosa* genome copies/mL in the PCD group compared to the Idiopathic/Post infective one, with respectively 172437673.1 (96693213.3-261253462.6) and 16875000.0 (95741.0-124480609.4) genome copies/mL, p=0.03.

No difference emerged in pathogens detection, and between genome copies levels of *S. pneumoniae*, *S. aureus* and *H. influenzae* (Table 3).

Table 3. Comparison of respiratory pathogens detection and quantification according to the study groups

Variable	PCD	Idiopathic/Post infective	p-value
P. aeruginosa detection	8 (40.0)	8 (40.0)	1.00
P. aeruginosa genome copies/mL	172437673.1 (96693213.3-261253462.6)	16875000.0 (95741.0-124480609.4)	0.03
S. pneumoniae detection	8 (40.0)	10 (50.0)	0.52
S. pneumoniae genome copies/mL	1984090.9 (153011.4-43312500.0)	3301.1 (2133.5-483806.8)	0.10
S. aureus detection	6 (30.0)	8 (40.0)	0.51
S. aureus genome copies/mL	53696.0 (3664.9-263496677.7)	75124.58 (3521.6-34032392.0)	1.00
H. influenzae detection	5 (25.0)	10 (50.0)	0.10
H. influenzae genome copies/mL	1576923.1 (148205.1-1048717948.7)	3546153.9 (11022.4-85801282.1)	0.59

3.2.1.3.3 Neutrophilic inflammation

Sputum neutrophilic inflammation analysis reported no statistical difference among the two groups (Table 4).

Table 4. Comparison of neutrophilic inflammation biomarkers according to the study groups

Variable	PCD	Idiopathic/Post infective	p-value
Active neutrophil elastase µg/mL	17.6 (5.1-31.3)	11.2 (6.2-31.7)	0.78
TNFα ng/mL	806.6 (132.2-1607.0)	1114.5 (634.6-1542.8)	0.44
IL6 ng/mL	265.8 (13.9-513.9)	149.7 (47.6-214.5)	0.33
IL1β ng/mL	367722.6 (203093.8-564737.2)	460710.9 (265215.3-716625.5)	0.24
IL10 ng/mL	28444.2 (9799.0-82130.4)	42415.1 (19518.7-60081.2)	0.60

3.2.1.4 Discussion

Data from this study identified a statistical increase in *P. aeruginosa* load in sputum of PCD patients. No differences in microbiome and inflammatory biomarkers were detected among PCD and idiopathic or post infective bronchiectasis.

P. aeruginosa is a well know pathogen colonizing bronchiectasis lungs and chronic infection by *P. aeruginosa* was associated to poor outcomes in bronchiectasis. Moreover, the increase in *Pseudomonas* is visible in compositional analysis; however, these data did not result to be significant. Furthermore, increased levels of *P. aeruginosa* are usually associated with an increase of active neutrophil elastase and low sputum diversity. Even if these data did not result to be statistically significant, results of alpha and beta diversity analysis show a clear trend. Chapter 4.1 of this thesis associated PCD aetiology to a decrease in alpha diversity. We can speculate that, even if the number of patients is similar to those published by Rogers and Colleagues, the low numbers associated to a rare disease did not allow us to completely unravel the association between PCD, microbial community and inflammation in bronchiectasis [1].

PCD is a rare disease and the enrolment of patients with PCD is very challenging. For this reason, the monocentric design seems not to be suitable to the study. This preliminary study lead us to the conclusion that a multicentric study is needed to efficiently study microbiome and inflammation in PCD. Starting from these data and from chapter 4.1 of this thesis, a new and more complete project with similar inclusion/exclusion and matching criteria, including PCD patients from Europe, is in preparation, increasing the sample size and enlarging the study from monocentric to multicentric. This solution will both increase the statistical power of our evaluations and the multicentric nature will enlarge the representability of data.

From these preliminary data, we can conclude that there is a trend in microbiome diversity along with statistical *P. aeruginosa* increase in PCD, however a larger study is needed to fully address this research question.

3.2.2 Immunodeficiency

3.2.2.1 Introduction

The interplay among immunological system and microbiome is fundamental in respiratory diseases. Experiences in literature reported a central role of gut microbiome in training host immunity. Recently the scientific community identified a similar role of lung microbiome in shaping local immunity. Immunodeficiency has been recognised as one of the aetiologies of bronchiectasis. A recent study demonstrated a difference in microbiome between aetiologies, including immunodeficiency. However, no specific data on microbiome in this group are available in literature.

The aim of this work was to investigate the association between immunodeficiency as an aetiology of bronchiectasis, microbiome and local inflammation.

3.2.2.2 Materials and Methods

3.2.2.2.1 Study design and population

A secondary analysis of the biobank and the clinical database of the Bronchiectasis Program of Respiratory Department, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy was conducted. Adults (≥18 years) referring to Bronchiectasis Program between March 2017 and March 2019 and with either primary or secondary immunodeficiency or idiopathic aetiology were included in the study. All patients underwent the same comprehensive immunological screening including complete blood count (CBC), serum electrophoresis, IgA, IgG, IgM, IgG subclasses, total IgE, lymphocytes subsets, and HIV antibodies assay. Clinical characteristics and past medical history to exclude differential diagnosis of hypogammaglobulinemia and secondary immunodeficiency conditions. Patients with at least one positive result for autoantibodies were evaluated by a rheumatologist to exclude an autoimmune disease. Bronchiectasis etiology was evaluated following the recommendations of the 2017 European Respiratory Society guidelines [1].

3.2.2.2.2 Study procedures

Clinical data, along with microbiome and neutrophilic inflammation ones were analysed and reported according to methods previously described in this thesis (Section "Sputum Neutrophil Elastase associates with microbiota and *P. aeruginosa* in bronchiectasis"). Patients were divided based on their aetiology: primary immunodeficiency, secondary immunodeficiency or idiopathic bronchiectasis.

3.2.2.3 Results

Sputum samples of 150 bronchiectasis patients [Female 110 (75.3%); Median (IQR) Age 63 (53.3-71)], 90 idiopathic, 56 primary and 4 secondary immunodeficiencies were collected in the study period. We decided to exclude from this analysis secondary immunodeficiency patients due to the low numbers.

Patients in the two groups resulted to be similar in terms of all the considered variables, Table 1.

Table 1. Description of the study population according to the study groups.

Variable	Idiopathic	Primary immunodeficiency	p-value
Sex (female), n (%)	69 (76.7)	41 (73.2)	0.78
Median (IQR) age, years	62 (51.5-71.0)	65 (57.8-73)	0.15
Former or current smoker, n (%)	45 (50.0)	21(37.5)	0.19
Median (IQR) FEV_1%predicted	83(67-95)	79.5(66.8-94)	0.65
Median (IQR) Bronchiectasis severity index	7 (5-10)	8(5-10)	0.53
Median (IQR) Exacerbations in the previous year	2(1-3)	2(1-3)	0.58
≥ 1 hospitalization in the previous year, n (%)	12(13.5)	8(14.3)	1.00
Inhaled antibiotics, n (%)	3 (3.3)	2 (3.6)	0.94
Azithromycin, n (%)	7 (8.0)	1 (2.0)	0.26

Specific description of primary immunodeficiency diagnosis is reported in table 2. Only 4of the patients underwent specific treatment for immunodeficiency after multidisciplinary discussion with the immunologist.

Table 2. Description of specific primary immunodeficiency diagnosis.

Primary Immunodeficiency	*N 56*
Unclassified immunodeficiency, n (%)	35 (62.5)
Isolated IgG subclasses deficiency, n (%)	6 (10.7)
Unclassified antibody deficiency, n (%)	7 (12.5)
Isolated IgM deficiency, n (%)	7 (12.5)
Common variable immunodeficiency, n (%)	1 (1.8)

3.2.2.3.1 Microbiome in primary immunodeficiencies

Compositional results are reported in Figure 1. Within-sample diversity was significantly different in idiopathic vs. primary immunodeficiencies with respectively median (IQR) Shannon 4.7 (3.5-5.7) vs.3.9 (2.7-5.1), p-value=0.02; Evenness 0.8 (0.7-0.9) vs. 0.7 (0.5-0.8), p-value=0.047 and Richness 63 (47-88.5) vs. 52 (26-73.5), p-value=0.008, Table 2.

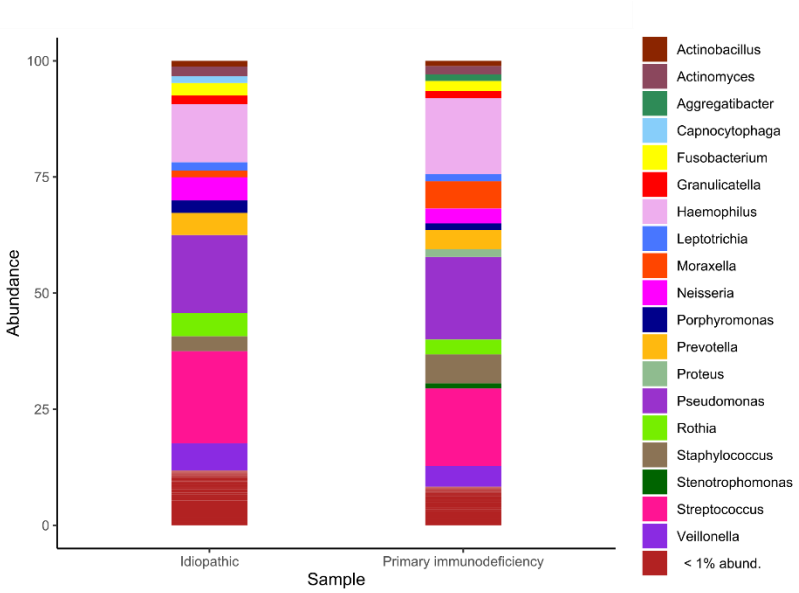
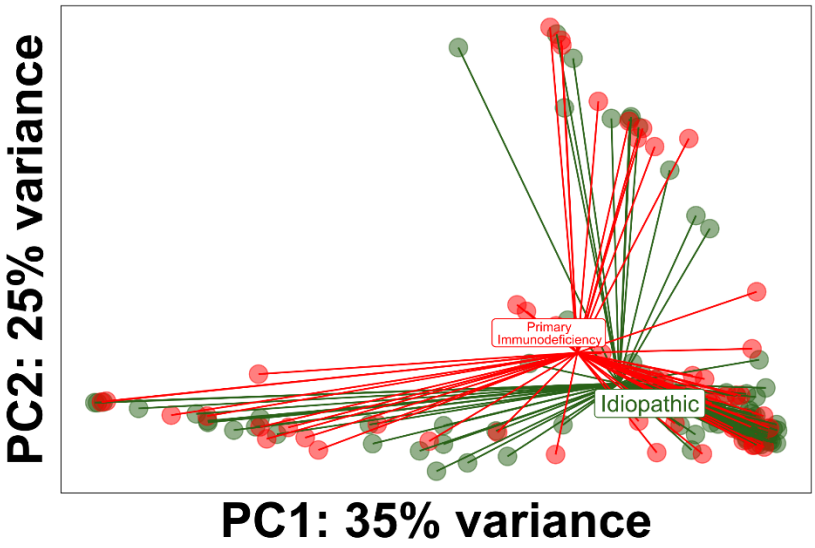


Figure 1. Genera Barplots in idiopathic and primary immunodeficiency groups.

Analysis on beta diversity (inter-sample diversity)evaluated through PCoA of Bray-Curtis dissimilarity did not report clustering (p=0.084) among the two study groups, Figure 2.

Figure 2. PCoA according to Bray-Curtis dissimilarity in idiopathic and primary immunodeficiency groups



3.2.2.3.2 Molecular detection and quantification of respiratory pathogens in sputum of patients with primary immunodeficiencies

However, no difference in the detection of *H. influenzae* was detected in sputum of idiopathic and primary immunodeficiency patients, a statistical increase of log genome copies/mL of *H. influenzae* in primary immunodeficiency patients was observed (Median (IQR) Idiopathic 5.1 (4.2-8.4) vs immunodeficiency 8.6 (5.6-9.1) log genome copies/mL, p-value=0.007). No difference was reported in detection and quantification of *P. aeruginosa*, *S. aureus* and *S. pneumoniae,* Table 3.

3.2.2.3.3 Sputum inflammation of patients with primary immunodeficiencies

No difference among idiopathic bronchiectasis and primary immunodeficiency was found according to inflammatory biomarker studied, Table 2.

Table 2. Biological characteristics of the study population according to the study groups represented as median (IQR) for continuous variables and n (%) for categorical ones.

	Idiopathic	**Primary immunodeficiency**	**p-value**
Alpha diversity			
Median (IQR) Richness	63 (47-88.5)	52 (26-73.5)	0.008
Median (IQR) Evenness	0.8 (0.7-0.9)	0.7 (0.5-0.8)	0.047
Median (IQR) Shannon	4.7 (3.5-5.7)	3.9 (2.7-5.1)	0.02
Molecular detection of respiratory pathogens			
P. aeruginosa detection, n (%)	37 (41.1)	28 (50)	0.38
Median (IQR) *P. aeruginosa* genome copies/mL, log	7.6 (7-8.2)	7.6 (6.9-8.1)	0.98
H. influenzae detection, n (%)	29 (32.2)	15 (26.8)	0.61
Median (IQR) *H. influenzae* genome copies/mL, log	5.1 (4.2-8.4)	8.6 (5.6-9.1)	0.007
S. aureus detection, n (%)	35 (38.9)	18 (32.1)	0.52
Median (IQR) *S. aureus* genome copies/mL, log	4.3 (3.4-6.8)	5.5 (3.6-7.8)	0.37
S. pneumoniae detection, n (%)	35 (38.9)	21 (37.5)	1.00
Median (IQR) *S. pneumoniae* genome copies/mL, log	3.5 (3.3-4.6)	3.6 (2.9-5.5)	0.66
Local inflammation			
Median (IQR) aNE, µg/mL	11.9 (5.1-31.3)	16.3 (9.8-30.3)	0.12
Median (IQR) TNFa, pg/mL	946.8 (542.4-1271.4)	929.9 (510.2-1188.2)	0.80
Median (IQR) IL-1B, ng/mL	284.1 (190.9-459.8)	362.8 (245.6-594.7)	0.18
Median (IQR) IL-6, pg/mL	146.3 (48.6-238.3)	130 (65.9-228.6)	0.67
Median (IQR) IL-10, ng/mL	23.7 (9.9-38.9)	21.3 (15.1-37.5)	0.85

3.2.2.4 Discussion

The main finding of this study is the identification of a less diverse sputum microbiome in terms of intra-patients diversity in adult patients with primary immunodeficiency compared to idiopathic bronchiectasis.

Patients with primary immunodeficiencies showed a decreased alpha-diversity in terms of sputum microbiome. Sputum dysbiosis is a typical condition in chronic respiratory diseases and decreased alpha diversity was described in severe bronchiectasis patients, in terms of respiratory function, radiological scores and inflammation [2–5]. Considering the immunological *disequilibrium* of patients with primary immunodeficiency, the identification of a perturbed diversity may be both associated to patients' clinical condition (radiology/respiratory function etc.) and to the immunodeficiency itself. As we mentioned the correlation among lung microbiome and immune system is very strict, we can speculate that an altered immune system may influence changes in microbiome and vice versa. However, studies unravelling the mechanisms underlying this relationship will be needed to deeply understand this association.

Moreover *H. influenzae* is a frequent colonizer of bronchiectasis airways and the higher detection of this pathogen may be related to the dysbiosis detected in primary immunodeficiencies lungs. No differences were found in terms of *P. aeruginosa* detection and quantification.

Although we considered a large immunological screening, future studies may include an even larger one considering second level analysis.

Patients' characterization is fundamental for precision medicine approach in bronchiectasis. These preliminary findings may pave the way to a more structured experience, evaluating the difference in microbiome and inflammation among both primary and secondary immunodeficiency and idiopathic bronchiectasis. Moreover, specific studies will be needed in order to understand the role of immunodeficiency in airway microbiome dysbiosis.

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Chapter 4

4 Airway neutrophilic inflammation and microbiome in bronchiectasis

4.1 Sputum Neutrophil Elastase associates with microbiota and *P. aeruginosa* in bronchiectasis

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Take-home message:

Active neutrophil elastase correlates with low microbiota diversity and *P. aeruginosa* detected as relative abundance (*Pseudomonas*), standard culture and targeted real time PCR in sputum samples of adult bronchiectasis patients in stable state.

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4.1.1 Introduction

Bronchiectasis is a chronic respiratory disease characterized by an abnormal and permanent dilatation of the bronchi in the context of a clinical syndrome of cough, sputum production, and frequent respiratory infections. [1] Neutrophilic airway inflammation is recognized as one of the major drivers of bronchiectasis pathophysiology and has clear implications on clinical outcomes. [2, 3] Up to 80% of bronchiectasis patients enrolled across different experiences worldwide show neutrophilic airway inflammation. [3–6] Neutrophil elastase (NE) detected in sputum is a promising biomarker in bronchiectasis patients, and its activity correlates not only with disease severity, lung function, and quality of life, but also with the exacerbation rate. [3]

NE is released from neutrophils during degranulation and formation of extracellular traps and has antibacterial effects against respiratory pathogens. [7] Among the different inflammatory stimuli driving the release of NE from neutrophils, bacteria and, specifically, Gram-negative infections (e.g., *Pseudomonas aeruginosa*, *Haemophilus influenzae,* and *Enterobacteriacee*) are the most potent.[3] Although the association between the activity of NE (aNE) and the presence of chronic bacterial infection has been widely explored through culture-based microbiology in both bronchiectasis and other chronic respiratory diseases, a deep analysis of the association of airways microbiota and neutrophilic inflammation measured by NE is still unreported in bronchiectasis. [3] Multiple studies enrolling patients with asthma, chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis or cystic fibrosis (CF) demonstrated a loss of bacterial diversity in the lung compared to healthy controls. [8–10] Experiences evaluating airway microbiota did not identify differences in alpha diversity between bronchiectasis patients and healthy subjects, although these results are limited by the small sample size and the large heterogeneity that characterizes this disease. [11–14]

We, therefore, postulated that bronchiectasis patients with elevated aNE comprised a more homogenous group characterized by a distinct microbiome with lower diversity.

4.1.2 Materials and Methods

4.1.2.1 Study design and population

An observational, cross-sectional study was carried out at Bronchiectasis Program of Respiratory Department, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, between March 2017 and March 2019. Consecutive patients aged ≥18 years with clinically (daily sputum production) and radiologically significant bronchiectasis (at least one lobe involvement on chest CT) were recruited during clinical stability (minimum one month from the last exacerbation and antibiotic course). Patients with CF or traction bronchiectasis due to pulmonary fibrosis were excluded. The study was approved by the ethical committee of the hospital, and all subjects provided written informed consent to participate.

4.1.2.2 Study procedures

Bronchiectasis patients underwent clinical, radiological, microbiological and functional evaluation. Spontaneous sputum samples were obtained and mucous plugs were isolated. DNA was extracted according to a published technique [15, 16]. Positive and negative controls were extracted along with samples. A second aliquot of mucous plug was diluted 8X in PBS, vortexed and centrifuged at 4°C for 15 minutes at 3,000g. Samples supernatants were stored at -80°C. For aNE assessment ProteaseTag® Active Neutrophil Elastase Immunoassay (Proaxis, Belfast, UK) was used as per manufacturer's instructions. [17]

4.1.2.3 Bacterial Real-Time PCR and microbiome evaluation

Bacterial DNA was amplified and quantified using real-time PCR assay for *P. aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *H. influenzae*[18].

High-throughput sequencing of bacterial 16S rRNA-encoding gene amplicons (V3-V4 region) was performed according to previously published studies [15, 16]**.**Specific description of real time PCR procedure and microbiome analysis is reported in the online supplement.

4.1.2.4 Clinical variables

Demographics, comorbidities, disease severity, aetiology of bronchiectasis, respiratory symptoms, sputum evaluation, radiological, functional and biological characteristics in stable state were recorded, see online supplement.

4.1.2.5 Study groups

A priori we split patients into two groups based on the concentration of aNE (20µg/mL) defined by Chalmers *et al.* as being most associated with worse outcomes in bronchiectasis: aNE ≥20 µg/mL VS. aNE<20 µg/mL. [3]

4.1.2.6 Statistical analysis

Variables were collected in an *ad hoc* electronic form. Qualitative variables were summarized with absolute and relative (percentage) frequencies, whereas quantitative variables with medians (interquartile ranges, IQR). Qualitative variables were statistically assessed with chi-squared or Fisher exact test, when appropriate, whereas those for quantitative non-parametric variables with Mann-Whitney. aNE was correlated to continuous variables with Spearman correlation. A two-tailed p-value was considered statistically significant when less than 0.05. A univariate and multivariate linear regression analysis was carried out to assess the relationship between levels of aNE and epidemiological and microbiological variables based on beta coefficients and related 95% confidence intervals. The statistical software STATA version 15 (StatsCorp, Texas, US) was used for all statistical computations.

Sample size calculation was based on the assumption of a higher level of Shannon diversity in patients with a lower aNE. We assumed a mean difference of 0.5 (means of 2.8 and 3.3 inaNE ≥20 µg/mland aNE <20 µg/ml, respectively) and a common standard deviation of 1.19; the estimated sample size was 180, with an alpha error of 0.05 and a statistical power of 0.8.

16S rRNA gene sequences were analysed using the Quantitative Insights into Microbial Ecology (QIIME version 2.0 2019.07 release) pipeline for analysis of microbiome data. [19]Microbiome data are available in Sequence Read Archive (SRA), accession number PRJNA605315. Code and metadata utilized for analysis are available at: https://github.com/segalmicrobiomelab/bronchiectasis.NE. Complete information on bioinformatic and statistical analysis are reported in the supplementary material.

4.1.3 Results

4.1.3.1 Study population

A total of 185 patients (74.6% females; median [IQR] age: 63 [51-71] years) was enrolled during the study period. Clinical and microbiological variables are reported in Table 1 according to the two study groups. Only 5 patients (2.7%) of our cohort showed a aNE level below the lower limit of detection. Patients in aNE ≥20 µg/ml group showed higher disease severity, with higher amount of daily sputum production, greater sputum purulence, and lower FEV_1. More than 50% of the patients had chronic infection identified by culture, mainly due to *P. aeruginosa* (30.8%), methicillin-sensitive *S. aureus* (MSSA) (9.2%), and *H. influenzae* (8.1%). *P. aeruginosa* was also detected through real-time PCR in 43.2% of the cases, *H. influenzae* in 30.3%, *S. aureus* in 38.4% and *S. pneumoniae* in 36.2%. In terms of microbiome diversity, the median [IQR] Shannon index was 4.33 [3.06 - 5.60] and Evenness was 0.75 [0.59-0.87].

A total of 109 (58.9%) patients had a median aNE <20µg/mL, whereas 76 (41.1%) ≥20µg/mL.

Table 1. Clinical characteristics of the study population according to the two study groups

Demographics		**aNE< 20 µg/ml** (n= 109)	**aNE ≥20 µg/ml** (n= 76)	**p-value**
Male		29 (26.6)	18 (23.6)	0.65
Age, years		63 (48-72)	63 (53.5-70.5)	0.81
Smoking status	Never	57 (52.3)	47 (61.8)	0.18
	Former/Active	52 (47.7)	29 (38.2)	
Body mass index		21.8 (19-25)	21.0 (19-24)	0.15
Radiology				
Reiff score		4 (3-6)	4.5 (3-9)	0.10
Disease severity				
BSI		7 (4-10)	8 (5-12)	0.03
BSI risk class	Mild	34 (31.2)	16 (21.1)	0.09
	Moderate	40 (36.7)	24 (31.6)	
	Severe	35 (31.1)	36 (47.4)	
FACED		2 (1-3)	3 (2-4)	0.02
FACED risk class	Mild	70 (64.2)	33 (43.4)	0.02
	Moderate	29 (26.6)	31 (40.8)	
	Severe	10 (9.2)	12 (15.8)	
Comorbidity				
BACI		0 (0-3)	0 (0-0)	0.24
History of Pneumonia		73 (67.0)	47 (61.8)	0.47
Gastro-esophageal reflux disease		48 (44.0)	33 (43.4)	0.93

Rhinosinusitis	43 (35.5)	25 (32.9)	0.36
Cardiovascular diseases	33 (30.3)	27 (35.5)	0.45
Systemic hypertension	24 (22.0)	16 (21.1)	0.88
Asthma	18 (16.5)	8 (10.5)	0.25
Immunodeficiency	17 (15.6)	12 (15.8)	0.97
Osteoporosis	14 (12.8)	13 (17.1)	0.42
Nasal polyposis	14 (12.8)	10 (13.2)	0.95
Previous neoplastic disease	12 (11.0)	14 (18.4)	0.15
COPD	11 (10.1)	7 (9.2)	0.84
Depression	8 (7.3)	9 (11.8)	0.30
History of Tuberculosis infection	8 (7.3)	6 (7.9)	0.89
Anxiety	7 (6.4)	4 (5.3)	1.0
Diabetes	5 (4.6)	1 (1.3)	0.40
Pulmonary hypertension	4 (3.7)	3 (4.0)	1.0
Atrial fibrillation	4 (3.7)	4 (5.3)	0.72
Other connective tissue disease	3 (2.8)	1 (1.3)	0.65
Rheumatoid arthritis	2 (1.8)	1 (1.3)	1.0
Stroke	1 (0.9)	1 (1.3)	1.0
Chronic renal failure	1 (0.9)	2 (2.6)	0.60
Etiology			
Idiopathic	44 (57.9)	68 (62.4)	
Primary ciliary dyskinesia	9 (11.8)	11 (10.1)	
Immunodeficiency	9 (11.8)	14 (12.8)	0.95
Post infective	7 (9.2)	9 (8.3)	
Other*	7 (9.2)	7 (6.4)	
Clinical status			
sputum volume	10 (5-25)	25 (7-75)	0.0003
>1 hospitalization previous year	10 (12.8)	14 (19.7)	0.25
Lung function			
Mean (SD) FEV_1, %predict.	80.6 (21.6)	76.4 (27.3)	0.25
FEV_1<50%predict.	6 (5.8)	14 (19.4)	0.005
Standard Microbiology			
Chronic infection	41 (40.2)	49 (67.1)	<0.0001
Chronic infection *P. aeruginosa*	24 (23.5)	33 (45.2)	0.003
Chronic Infection MSSA	10 (9.8)	7 (9.6)	0.96
Chronic Infection *H. Influenzae*	11 (10.8)	4 (5.5)	0.28
Chronic Infection *S. pneumoniae*	1 (1.0)	1 (1.4)	1.0
Chronic Infection MRSA	1 (1.0)	1 (1.4)	1.0
Chronic Infection *S. maltophilia*	5 (4.9)	0 (0.0)	0.08
Chronic Infection *Achromobacter*	0 80.0)	3 (4.1)	0.07
Other chronic infection	4 (3.9)	3 (4.1)	1.0

Chronic Infection *A. fumigatus*	1 (1.0)	0 (0.0)	1.0
Molecular Biology			
Real Time PCR for *Pseudomonas* spp.	35 (32.1)	45 (59.2)	<0.0001
Pseudomonas spp., copies/ml, $\log_{10}$	6.9 (5.8-7.5)	8.0 (7.5-8.3)	<0.0001
Real Time PCR for *H. influenzae*	40 (36.7)	16 (21.1)	0.02
H. influenzae, copies/ml, $\log_{10}$	5.0 (4.3-8.5)	8.4 (4.7-9.0)	0.16
Real Time PCR for *S. aureus*	47 (43.1)	24 (31.6)	0.11
S. aureus, copies/ml, $\log_{10}$	4.6 (3.5-7.6)	4.0 (3.3-7.2)	0.36
Real Time PCR for *S. pneumoniae*	44 (40.4)	23 (30.3)	0.16
S. pneumoniae, copies/ml, $\log_{10}$	3.6 (3.2-5.5)	3.3 (2.7-4.7)	0.13

Definition of abbreviations: BSI= Bronchiectasis severity index; MRSA= Methicillin-Resistant *Staphylococcus Aureus*; MSSA= Methicillin- sensitive *Staphylococcus Aureus*; COPD = chronic obstructive pulmonary disease;
Data are presented as median (interquartile range) or n (%).

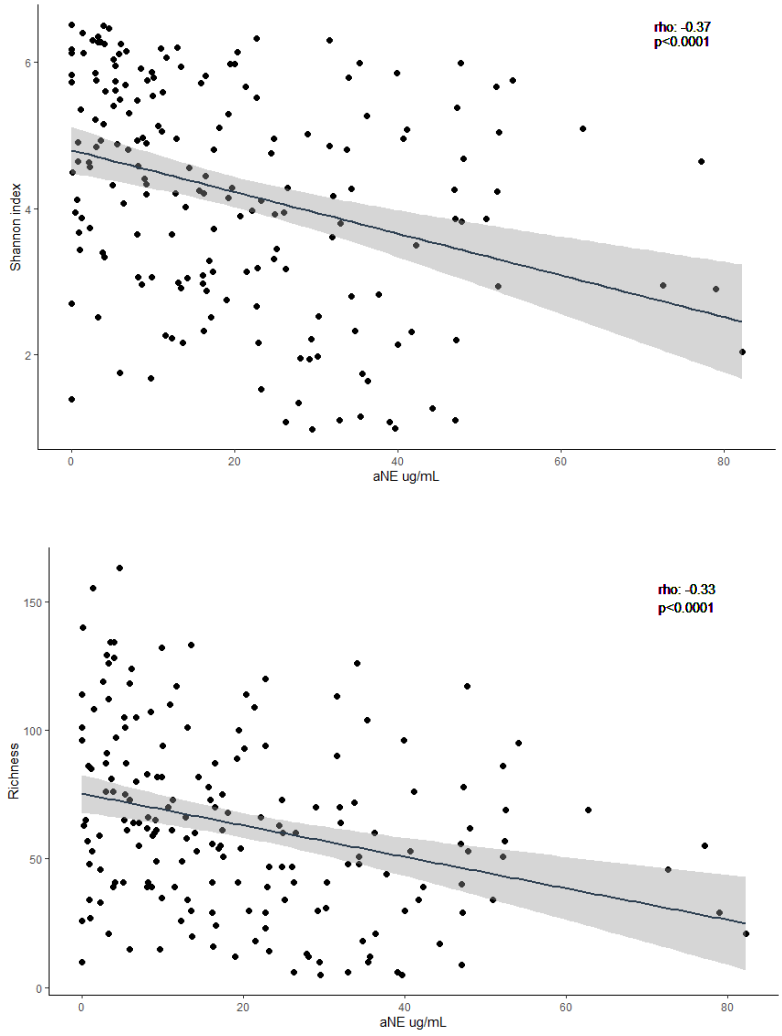
* Other includes COPD, Connective Tissue Diseses, Alpha1-antitrypsin deficiency, ABPA, Asthma, CFTR-RD, Aspiration

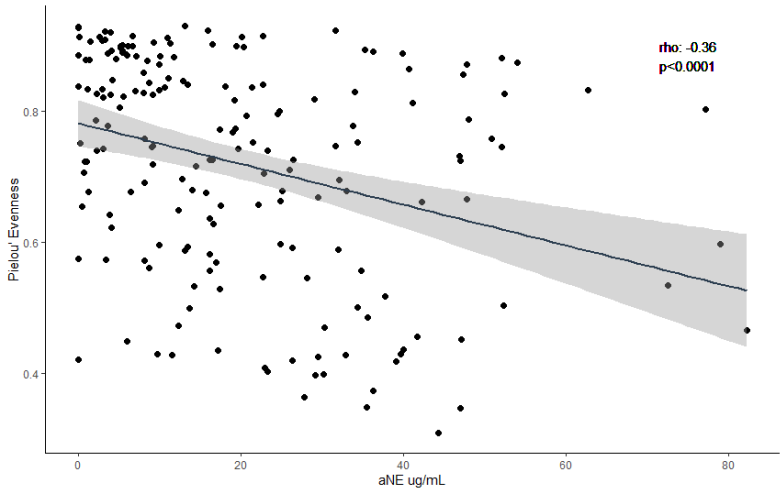
4.1.3.2 Microbiome and NE activity

Positive and negative controls were sequenced along with samples. Negative control was reported to have 210 reads that were filtered out during the quality step. Positive control showed *Bacillus* 24%, *Staphylococcus* 12%, *Lactobacillus* 12%, *Enterobacteriaceae* 11%, *Escherichia* 11%, *Salmonella* 11%, *Listeria* 10%, and *Pseudomonas* 9%.

Within-sample microbial diversity, evaluated through the Shannon entropy (rho: -0.37; p-value<0.00001), Pielou' evenness (rho: -0.36, p-value<0.00001) and richness (rho: -0.33; p-value<0.00001), was inversely correlated with values of aNE (Figure 1).

Figure 1. Correlation between NE activity (aNE) and Shannon entropy, Pielou' evenness, and richness.

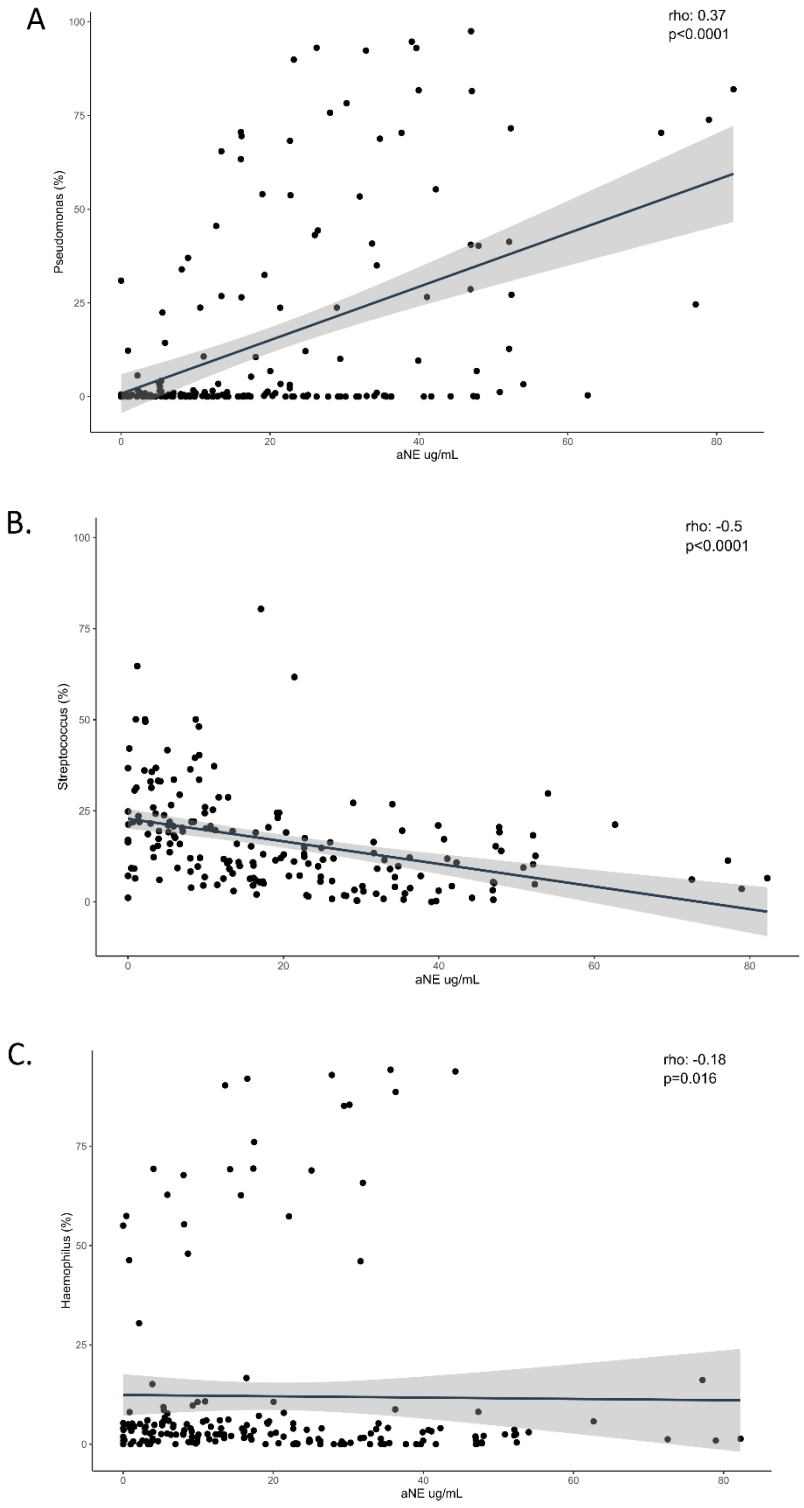


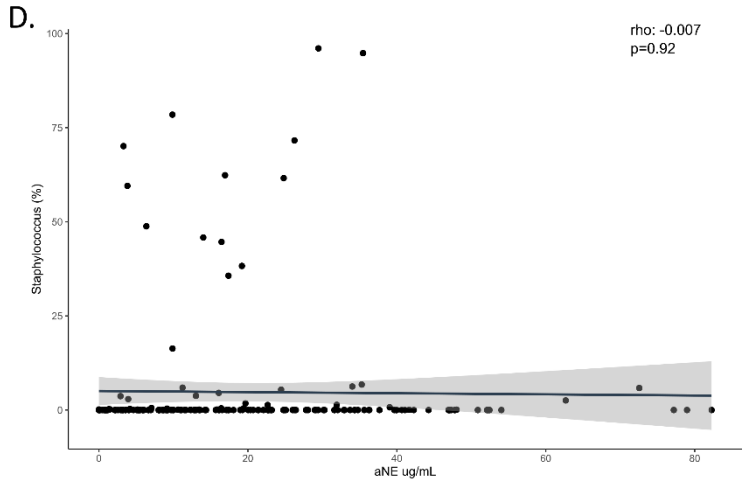


Moreover, similar results in within-sample diversity are shown considering the two study groups.

A significant difference in terms of median [IQR] levels of Shannon diversity was detected between patients with aNE ≥20 µg/mL [3.82 (2.20-4.96)] VS. aNE<20 µg/mL[4.88 (3.68-5.80)], p-value<0.0001 (Figure A online supplement). A significant difference was found between patients with aNE <20 µg/mL in terms of median [IQR] levels of Richness [aNE ≥20 µg/mL 47.0 (29-70.0) VS. aNE <20 µg/mL 65.5 (47.5-96.0); p-value<0.001] and Evenness [aNE ≥20 µg/mL 0.68 (0.47-0.81) VS. aNE <20 µg/mL0.82 (0.68-0.8878) p-value<0.0001] (Figures A online supplement).Similar results in Shannon diversity were obtained when PCD patients as well as those with primary/secondary immunodeficiency have been excluded (see table A online supplement). A direct correlation between aNE and Pseudomonas relative abundance was found (rho=0.37, p<0.0001), while, an inverse correlation is shown between Streptococcus, and aNE levels (rho=-0.5; p<0.001). No correlation between Staphylococcus and aNE while a mild inverse correlation emerged with Haemophilus (rho=-0.18; p=0.016), see figure 2.

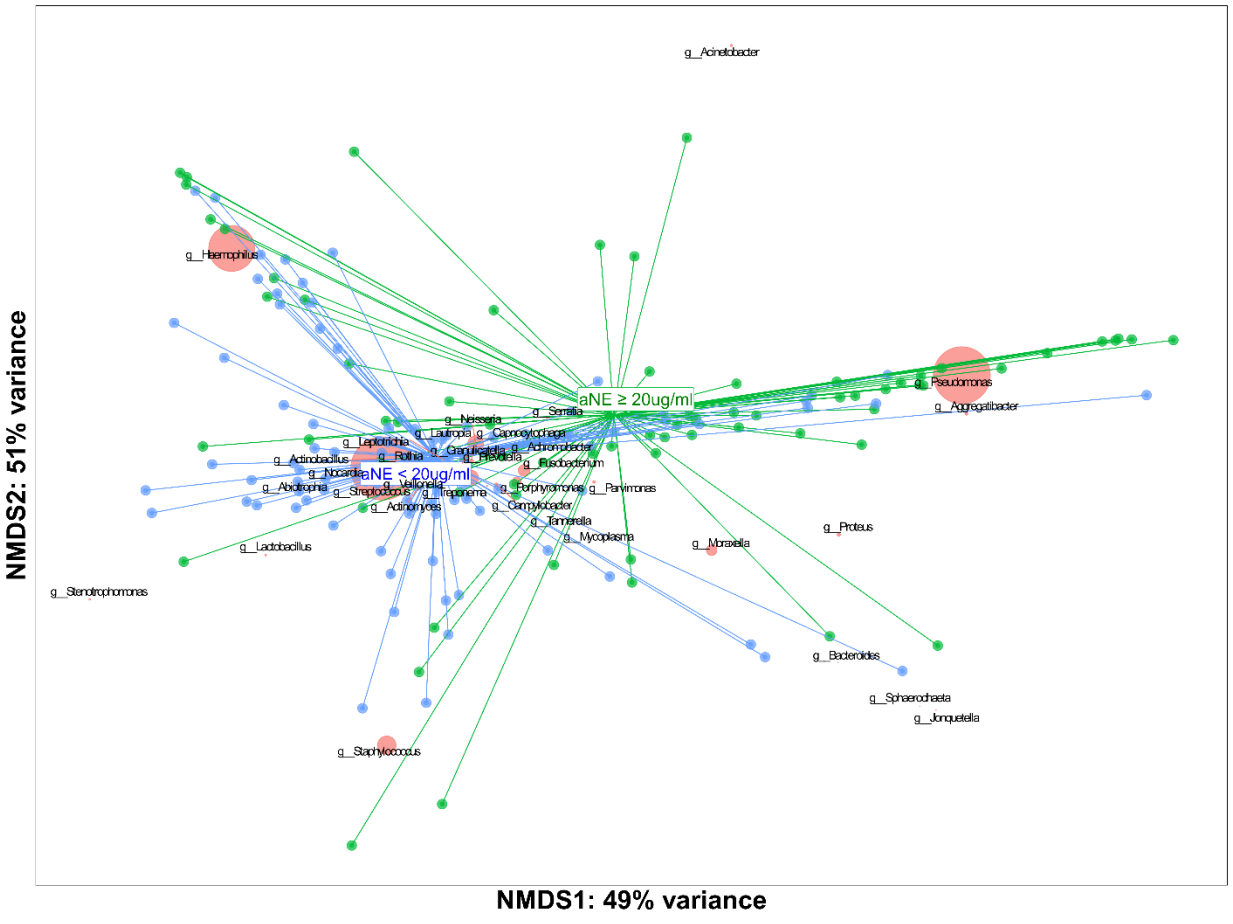
Figure 2. Correlation of aNE levels with *Pseudomonas* (A), *Streptococcus* (B), *Haemophilus* (C) and *Staphylococcus* (D) relative abundance.





Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity is shown in Figure 3. Samples clustered in two separate groups according to aNE levels (PERMANOVA p=0.001). Biplot of samples (categorized according to aNE levels) and taxa (summarized at a genus level) showed that the high aNE group co-occurred with *Pseudomonas* whereas the low aNE group co-occurred with *Streptococcus*, *Haemophilus*, and *Staphylococcus*.

Figure 3. Biplot including non-metric multidimensional scaling on the basis of Bray-Curtis distances for groups of aNE ≥20 µg/mL (green) and aNE <20 µg/mL (blue) and genera (pink)



Further compositional analysis showed a differential enrichment between the two groups: *Pseudomonas* was the only genus increased in the aNE ≥20 µg/mL group, while *Streptococcus*, *Rothia*, *Actinomyces, Abiotrophia* and *Atopobium* were differentially enriched among aNE <20 µg/mL samples, see volcano plot in Figure B in the online supplement.

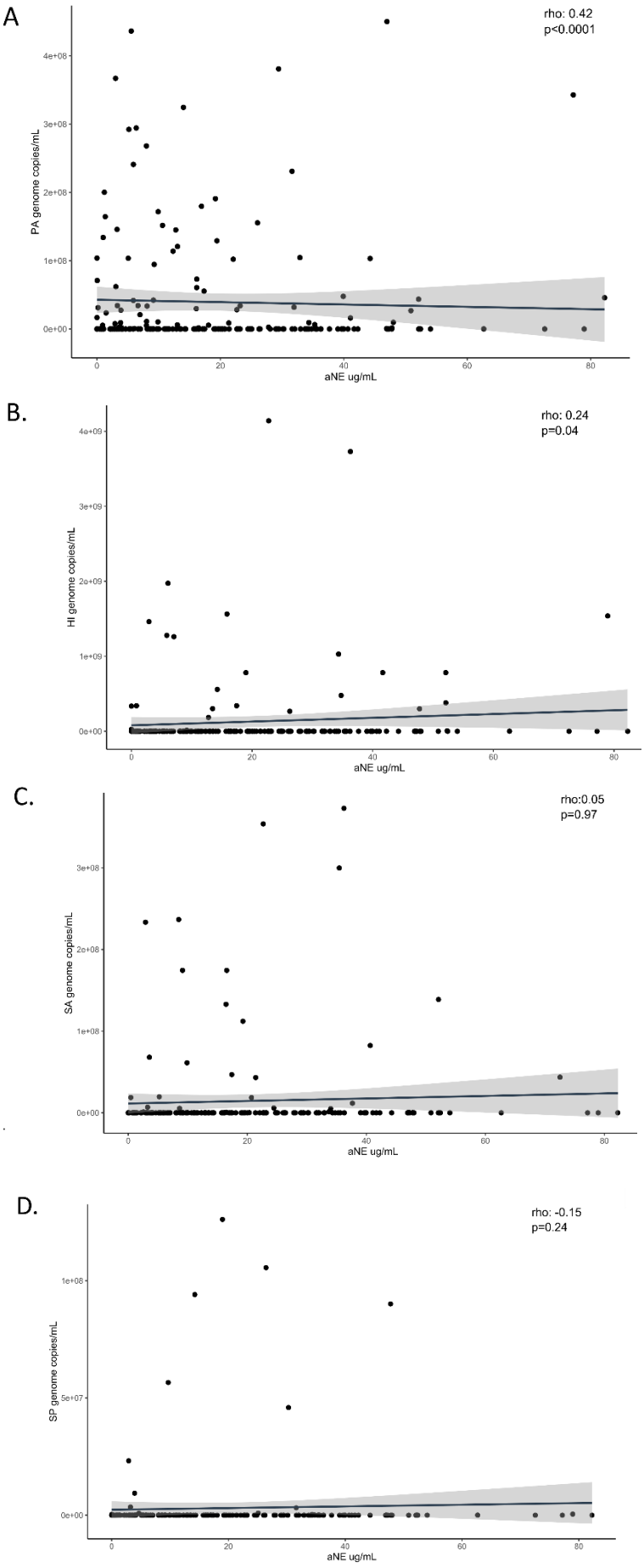
4.1.3.3 NE activity and chronic bacterial infection at standard microbiology

Chronic bacterial infection and chronic *P. aeruginosa* infection (defined by culture) were more prevalent in patients with aNE ≥20 µg/ml (chronic bacterial infection: aNE ≥20 µg/ml 67.1% VS. aNE <20 µg/ml 40.2%, p<0.0001; chronic *P. aeruginosa* infection, aNE ≥20 µg/ml 45.2% VS. aNE <20 µg/ml 23.5% p=0.003). No difference in the distribution of chronic infection by *S. aureus*, *H. influenzae*, *S. pneumoniae, Achromobacter denitifricians* was found between the two groups.

4.1.3.4 NE activity and molecular biology

A significant correlation was found between aNE and the number of copies/mL of *P. aeruginosa* DNA in sputum (rho: 0.42, p-value <0.0001). Patients with levels of aNE ≥20 µg/mL present with a higher real time PCR detection in sputum for *P. aeruginosa* (aNE ≥20 µg/ml 59.2% VS. aNE <20 µg/ml 32.1%, p<0.0001). No difference in distribution of infection by *S. aureus* and *S. pneumoniae* was found between the two groups and no correlation was found between aNE and number of copies/mL of DNA load for *S. aureus* and *S. pneumoniae*. At Real Time PCR, *H. influenzae* was significantly more detected in sputum with lower levels of aNE while a direct significant correlation was found between aNE and the number of copies/mL of *H. influenzae* DNA in sputum (rho: 0.24, p-value =0.04) (Figure 4). A multivariate analysis between *H. influenzae* and *P. aeruginosa* bacterial load is presented in table B of the online supplement. This multiparametric analysis showed that *H. influenzae* did not correlate with aNE levels. *P. aeruginosa* is the only significant variable associated with aNE [Beta (95%) confidence interval: *P. aeruginosa* 7.8 (0.7-14.9), p=0.04;*H. influenzae* 5.0 (-1.2-11.3), p=0.11].

Figure 4. Correlation between aNE and number of copies/mL of DNA load for *P. aeruginosa* - PA (Fig. A), *H. influenzae* – HI (Fig. B), *S. aureus* – SA (Fig. C), and *S. pneumoniae* - SP (Fig. D).


A
rho: 0.42
p<0.0001
PA genome copies/mL
4e+08
3e+08
2e+08
1e+08
0e+00
0
20
40
60
80
aNE ug/mL
B.
rho: 0.24
p=0.04
HI genome copies/mL
4e+09
3e+09
2e+09
1e+09
0e+00
aNE ug/mL
C.
rho:0.05
p=0.97
SA genome copies/mL
3e+08
2e+08
1e+08
0e+00
aNE ug/mL
D.
rho: -0.15
p=0.24
SP genome copies/mL
1e+08
5e+07
0e+00
aNE ug/mL

4.1.4 Discussion

The most important findings of the present study are: 1) Higher levels of aNE in sputum samples from bronchiectasis patients were correlated with a decreased microbiome diversity; 2) *Pseudomonas* as genus and *P. aeruginosa* as specie, detected through both molecular biology and culture, showed the best association with high levels of aNE; 3) In addition to the presence or absence of *P. aeruginosa* on both culture and molecular biology, high levels of aNE were correlated with the amount of *P. aeruginosa* genome copiesin sputum.

As new finding and in line with our hypothesis, we found an inverse correlation between microbiota diversity and levels of aNE in sputum (based on Shannon, Richness and Evenness indexes). This difference in terms of microbiota diversity was also confirmed by the evaluation of the beta diversity, which allowed separating samples in two well-defined groups. The correlation between aNE and a reduced microbiota diversity in sputum is a novel finding in bronchiectasis patients. This is consistent with studies in CF, where Zemanick *et al.* demonstrated that NE levels negatively correlated with microbiota diversity, and reported an association between NE levels and *P. aeruginosa* infection. [8]

From a clinical perspective, the evaluation of aNE in sputum of bronchiectasis patients confirmed data published by Chalmers and coworkers who underlined the correlation between aNE and both disease severity and lung function. [3] From a microbiome perspective, the characteristics of our population were very similar to those reported in previous experiences enrolling adults with bronchiectasis. For example, Lee *et al.* observed that the most abundant phyla among subjects with bronchiectasis are *Proteobacteria* and *Firmicutes*. [13] Furthermore, the median levels of aNE in sputum of our patients were very similar to those reported by Chalmers and coworkers in a Scottish population of bronchiectasis patients. [3]

We also confirmed the major role of *P. aeruginosa* in increasing neutrophilic inflammation in bronchiectasis. [20] In the current investigation, the role of *Pseudomonas* as genus and *P. aeruginosa* as specie was evaluated not only through microbiome and culture, but also through molecular biology techniques showing a positive correlation between *P. aeruginosa* genome copies/mL and aNE. These data support the hypothesis that *P. aeruginosa* appears to be the most potent bacterial stimulus of neutrophil recruitment and release of NE in the bronchiectasis airways. As a new finding, we also discovered that the increased aNE in patients with *P. aeruginosa* was strongly associated with the load of *P. aeruginosa* detected in sputum, opening a new perspective on the role of molecular biology in bronchiectasis. This finding is also in line

with recent data showing that bacterial load evaluated through standard microbiology is associated with worse quality of life and increased lung inflammation in bronchiectasis. [21] Further investigations on the role of molecular biology as an aid to define disease severity, outcomes and treatment responses are needed.

Real-time PCR data suggested that the presence of *H. influenzae* was associated with lower levels of aNE, although there was a mild direct correlation between genome copy number and aNE. *H. influenzae* is a Gram-negative bacterium and previous studies described a correlation between chronic infection with *H. influenzae* and higher levels of NE. [3]The increase observed in *H. influenzae* colonized samples seemed to be due to co-infection with *P. aeruginosa*. Multivariate analysis highlighted that *P. aeruginosa* seemed to be the driver of aNE levels even in presence of *H. influenzae*. [14]Our data suggest that the presence of a co-infection with *P. aeruginosa* and its burden are needed to increase levels of aNE in sputum.

With regards to the other genera, Zemanick et al. also described a correlation between *Staphylococcus*, and *Enterobacteriaceae* and higher levels of NE in CF while no association between NE levels and *Haemophilus*, *Prevotella*, and *Rothia* was detected [8, 12] We confirmed the increased enrichment of *Streptococcus* genus associated with low levels of aNE, whereas, no evidence of association with *Staphylococcus* was found.[22] Oral commensals are frequently found in healthy sputum microbiota, replaced in disease status with pathogenic bacteria. [23]

One of the possible implications of our study is that aNE levels in sputum provide significant insights into the microbial environment in the airways. Microbiota analysis for the assessment of microbial environment in lungs could be used in future clinical trials evaluating the effectiveness of NE inhibitors. Excess of proteases in lungs causes tissue damage and alters the remodeling process. In order to counteract this imbalance, NE inhibitors are currently under clinical development in bronchiectasis. [24–26] Previous and ongoing clinical trials on NE-inhibitors were not designed considering bacterial infection with specific pathogens or the presence of active elastase in sputum. The findings presented in the current investigation suggest that presence and burden of *P. aeruginosa*, and/or aNE levels could potentially be a useful biomarker where the impact of NE inhibitors could be greatest. [24, 25] Our data are consistent with Keir and colleagues and indicate that while patients with chronic *P. aeruginosa* infection may seem to be the best candidates for NE inhibition, its isolation might not be enough to presume high levels of aNE in sputum. [20] Further, the load of *P. aeruginosa* is emerging as a determining factor for the presence of high aNE levels in sputum of bronchiectasis patients and could therefore be used as a biomarker to direct NE inhibition.

One of the major limitations of this study includes the use of sputum as matrix of interest, which is not completely representative of the lower airways. However, sputum is a non-invasive easily accessible sample and would be preferable as a biomarker that can be repeated multiple times over invasive lower airway samples. Furthermore, we did not assess oral microbiota, a major contaminant of sputum. Future investigation should include oral sampling paired with sputum samples in order to dissect the topographical source of the microbial signatures identified. The monocentric design of this study might limit the generalizability in geographical areas where microbiology could be different. Moreover, viral, mycobacterial and fungal communities were not evaluated. The microbiota analysis through 16S rRNA gene sequencing constitutes a cost-effective approach but with limited capabilities due to lack of strain level resolution and functional assessment. In addition, our study did not take into account microbiota dynamics and future investigations are needed to both confirm our data and improve our findings with a longitudinal assessment during stable state and exacerbation.

Microbial functioning may affect host immune response and aNE levels should also be investigated to better explore host-pathogen interaction in bronchiectasis. In conclusion, aNE levels are inversely associated to within-sample diversity in sputum of bronchiectasis patients, and directly proportional to *P. aeruginosa* relative abundance and load, underling the importance of the assessment of the whole community.

Considering the clinical need for treatment of bronchiectasis patients, it is helpful to understand the microbial environment in lungs, which could both serve as a biomarker for trials on NE inhibitors or a novel target for personalized approaches. In conclusion, aNE levels are inversely associated to within-sample diversity in sputum of bronchiectasis patients, and directly proportional to *Pseudomonas* relative abundance and *P. aeruginosa* load. The identification of the association between *P. aeruginosa* and aNE and the decreased alpha diversity suggest the microbiome to be a possible target for future treatments directly targeting lung microbiota.

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4.1.6 Online data supplement

4.1.6.1 Methods

4.1.6.1.1 Microbiome evaluation

High-throughput sequencing of bacterial 16S rRNA-encoding gene amplicons (V3-V4 region) was performed according to previously published studies. [1, 2] Briefly, DNA extraction was performed using Roche High Pure PCR Template Preparation Kit (Hoffmann – La Roche. Basilea. Switzerland) with a pretreatment with dithiothreitol (DTT; Sputafluid, Biolife Italiana Srl, Italy) and enzymatic lysis with 3.6 mg/ml lysozyme and 0.18 mg/ml lysostaphin (Sigma-Aldrich. Saint Louis. Missouri, USA). Reagent control samples and mock mixed microbial DNA were sequenced and analyzed in parallel. V3-V4 16S rRNA gene were amplified from DNA extracts using the 16S metagenomic sequencing library preparation protocol (Illumina, San Diego, CA, USA) using suggested primers. Amplicons were barcoded using Nextera XT library prep kit (Illumina, San Diego, CA, USA) as per manufacturer's instructions. PCR products, approximately sized 630 base-pairs, were visualized using microfluidics-based gel electrophoresis on Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA) and, then, were cleaned using AMPure XP magnetic bead-based purification (Beckman Coulter, Brea, CA, USA). [1, 2] Sample libraries were quantified using Qubit and, then, pooled in an equimolar mode. Finally, the pool was sequenced on the MiSeq (Illumina, San Diego, CA, USA) sequencing platform, using a 2 _ 300 cycle V3 kit and following standard Illumina sequencing protocols.

4.1.6.1.2 Real time PCR for bacterial detection

Bacterial DNA was amplified and quantified using a real-time PCR assay with TaqMan probes for *P. aeruginosa* (gyrB gene), *Staphylococcus aureus* (nuc gene), *Streptococcus pneumoniae* (lytA gene), and *H. influenzae* (fucK gene) using AB7900HT Fast Real-Time PCR System (Applied Biosystems), with primers and probes previously published. [3]

Quantitative PCR amplification was performed in 20-µL reaction mixture containing 2x QuantiFast Multiplex PCR Master Mix (Qiagen), primers and probes and 2 µL of DNA extracted from sputum samples. Moreover, a real-time PCR targeting human RNaseP gene was used to detect PCR inhibition or extraction failure. Amplification was performed with the following parameters: 95°C for 5 minutes, followed by 45 cycles of 95°C for 45 seconds and 60°C for 1 minute. To quantify the amount of bacterial DNA in each sample standard curves were prepared using quantitative genomic DNA from S. pneumoniae (ATCC® 700669DQTM), *P. aeruginosa* (ATCC® 47085DQTM), S. aureus (ATCC® 29213DQTM), and H. influenzae (ATCC® 51907DQTM). These were quantified with Qubit and Quant-iT dsDNA Assay Kit High

Sensitivity and, then, diluted at 1ng/µL; subsequently, standard curve was prepared by 10-fold dilution from 1ng/µL to 1*10-7 ng/µL. Samples and controls were tested in triplicate and samples were assumed to be positive if Ct is <38. Target genes (gyrB, nuc, lytA and fucK) were in a single copy in the genome and the number of measured copies was assumed to be equivalent to the bacterial load. [4] The number of genome copies were calculated based on 7.22 fg, 3.01 fg, 2.28 fg, and 1.95 fg of DNA per *P. aeruginosa*, *S. aureus*, *S. Pneumoniae*, and *H. influenzae* genomes, respectively. Quantification of bacteria found in each sample was based on standard curves generate by plotting the Ct values against known genome copies. Conversion of genome copies/reaction to genome copies/mL was based on a 2µL input per reaction derived from 100 µL of eluate extracted from 200 µL of treated specimen.

4.1.6.1.3 Clinical variables

The severity of bronchiectasis was evaluated according to both the Bronchiectasis Severity Index (BSI) and FACED score (evaluating FEV_1, Age, Chronic infection with *Pseudomonas*, Radiological Extension and Dyspnea). [5, 6] The impact of comorbidities has been evaluated using the Bronchiectasis Aetiology Comorbidity Index (BACI). [7] Radiological severity of bronchiectasis was assessed using a modified Reiff score, which rates the number of involved lobes (with the lingula considered to be a separate lobe) and the degree of dilatation (range: 1-18). [8] All bacteriology was performed on spontaneous sputum samples as previously described. [9] Murray-Washington criteria for sputum quality was used in all cases, with all samples having less than 10 squamous cells and more than 25 leukocytes per low-power microscope field. Chronic infection was defined by the isolation of potentially pathogenic bacteria in sputum culture on two or more occasions, at least 3 months apart over a 1-year period. [10]

4.1.6.1.4 Bioinformatic analysis

The obtained 16S rRNA gene sequences reads were de-multiplexed and quality filtered with default parameters using the Quantitative Insights into Microbial Ecology (QIIME). For the QIIME2 pipeline, after demultiplexing, sequences were matched through the DADA2 pipeline to generate representative sequences and a frequency table consisting of amplicon sequence variants (ASV). [11, 12] Following DADA2, a quality control step was then used to filter out non-microbiota sequences with a 97% homology to a Greengenes database. Remaining sequences were assigned taxonomy based on 99% homology to a trained classifier with 99% homology to the V3-V4 region. [13] No OTU was removed from the analysis.

The proportion of reads at the ASV or genus levels were used as a measure of the relative abundance of each type of bacteria. Shannon Diversity Index on rarefied data was utilized to evaluate alpha diversity (within sample diversity). Weighted UniFrac was used to measure beta diversity (between sample diversity) of bacterial communities and to perform principal coordinate analysis (PCoA). We used the ade4 package in R to plot a PCoA on weighted UniFrac distances.[14]UniFrac distance was used for nonmetric multidimensional scale (NMDS) biplot evaluation of sample distribution and taxa (on relative abundance genus table pruned to include only genera with >5% relative abundance). [15]To evaluate differences between groups of 16S rRNA gene sequencing data, we used Differential gene expression analysis based on the negative binomial distribution the DESeq2 (v3.5, R Bioconductor) package. [16] Sequence data are available from the NCBI Sequence Read Archive (accession number to the bioproject PRJNA389299). All codes utilized for the analysis included in this manuscript are available at https://github.com/segalmicrobiomelab/bronchiectasis.neutrophil.elastase

4.1.6.2 Results

Table A: Shannon diversity (groups and correlation) analysis in different aetiology subgroups.

Study group	N	Median [IQR] aNE ≥20 µg/mL	Median [IQR] aNE <20 µg/mL	p (Mann-Whitney)	rho	p (Pearson's correlation)
Total population	185	3.82 (2.20-4.96)	4.88 (3.68-5.80)	<0.0001	-0.36	<0.00001
Total with neither PCD nor Immunodeficiency	139	3.84(2.56-5.00)	4.89 (3.95-5.80)	<0.0001	-0.354	<0.0001
PCD or Immunodeficiency	46	2.90 (1.95-4.18)	4.25 (2.97- 5.86)	0.011	-0.417	0.004

Table B: Multivariate analysis to assess the relationship between aNE, *P. Aeruginosa* and *H. influenzae* copies.

	Beta (95%) CI	p-value
Males	-5.7 (-0.27; 16.4)	0.59
Age, years	0.2 (-0.8; 1.2)	0.61
Median (IQR) *P. aeruginosa*, copies/ml, log10	7.8 (0.7-14.9)	0.04
Median (IQR) *H. influenzae*, copies/ml, log10	5.0 (-1.2; 11.3)	0.11

Figure A. Comparison of median levels of Shannon diversity, Evenness and Richness between the two study groups (aNE ≥20 µg/mL VS. aNE <20 µg/mL).

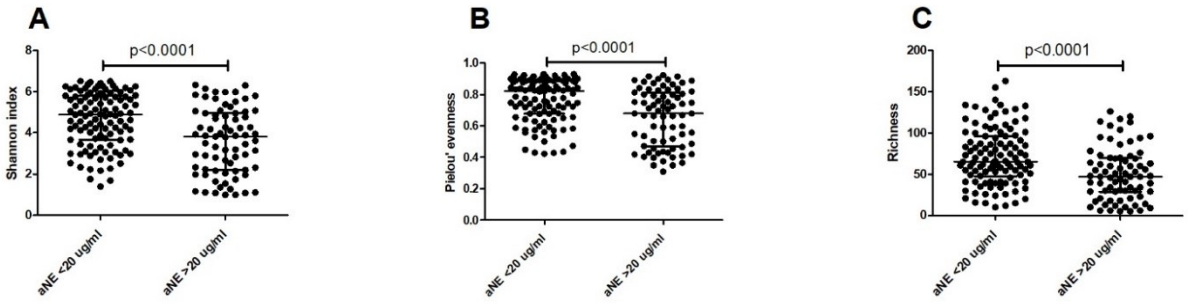
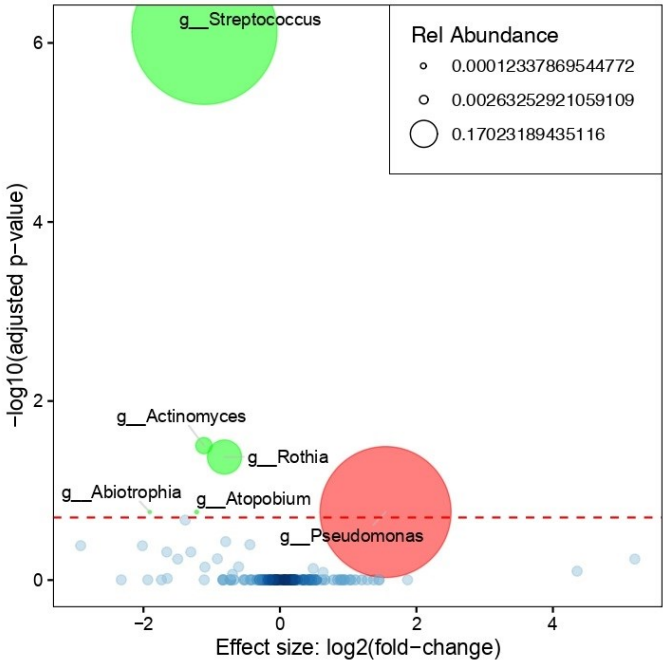


Figure B. Volcano plot showing a differential enrichment between aNE ≥20 µg/mL (red) and aNE <20 µg/mL (green).



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4.2 Sputum Cathepsin G levels and microbiome in bronchiectasis

4.2.1 Introduction

As reported in the general introduction, neutrophilic inflammation is central in bronchiectasis. Serine proteases are effectors of neutrophilic inflammation and their role in fighting bacterial infection and specifically gram-negative onesis known. Neutrophil elastase, a serin protease was reported to be a biomarker of disease severity and poor outcomes in bronchiectasis [1]. The broadly used laboratory tests evaluating neutrophil elastase were not able to distinguish among serine proteases [2]. The technical development of assays able to specifically assess the activity of each serine protease opened the way to the evaluation of the others serine proteases as new possible biomarkers for bronchiectasis [2]. Cathepsin G was reported to be actively contributing to tissue damage in lungs and pre-clinical experiences reported an interfering effect of cathepsin G in the clearance of *P. aeruginosa* [3]. Moreover, the interaction between cathepsin G and microbiome is still unknown.

In the context of a large international study we decided to run a sub-study investigating the interaction among active cathepsin G (Cat-G) and microbiome analysis.

4.2.2 Material and Methods

An observational, prospective study was carried out at Bronchiectasis Program of Respiratory Department, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, Ninewells Hospital, Dundee, United Kingdom, and Respiratory Department, Hospital de la Santa Creu i Sant Pau, Biomedical Research Institute Sant Pau (IIB Sant Pau), Barcelona, Spain between March 2017 and May 2019. Consecutive adult patients (age ≥18 years) with clinically and radiologically significant bronchiectasis were recruited during clinical stability (see study definitions). Exclusion criteria was having CF or traction bronchiectasis due to pulmonary fibrosis. The study was approved by the ethical committee of the hospital, and all subjects provided written informed consent to participate.

4.2.2.1 Study procedures

All bronchiectasis patients enrolled underwent clinical, radiological, microbiological and functional evaluation (see "Supplementary material" of Section "Sputum Neutrophil Elastase associates with microbiota and *P. aeruginosa* in bronchiectasis").

Patients enrolled provided a spontaneous sputum sample for Cat-G and aNE evaluation. Moreover, DNA extraction, supernatant production, bacterial Real-Time PCR and microbiome evaluation were carried out following the procedures described in "Supplementary material" of Section "Sputum Neutrophil Elastase associates with microbiota and *P. aeruginosa* in bronchiectasis" of this thesis. For Cat-G and aNE measurments, ProteaseTag® Active Cathepsin G Immunoassay (Proaxis, Belfast, UK) and ProteaseTag® Active Neutrophil Elastase Immunoassay (Proaxis, Belfast, UK) was used as per manufacturer's instructions. [4]

4.2.2.2 Study groups

Patients with undetectable Cat-G were excluded from the study. The remaining patients were split into three groups based on the tertiles of concentration of active Cathepsin G (Cat-G).

4.2.2.3 Statistical analysis

An *ad hoc* electronic form was used to collect specific variables. Absolute and relative (percentage) frequencies and medians (interquartile ranges, IQR) were respectively used to summarize qualitative and quantitative variables. Different statistical methods were used based on the nature of the variables. Chi-squared or Fisher exact test, were used on qualitative variables, whereas Mann-Whitney or Kruskall-Wallis test were used on quantitative ones when appropriate. Moreover, correlation of Cat-G with continuous variables was done using Spearman correlation. A two-tailed p-value lower than 0.05 was considered statistically significant. The statistical software STATA version 15 (StatsCorp, Texas, US) was used for all statistical computations.

No preliminary studies were found on cathepsin G in bronchiectasis. For this reason, the sample size was based on sample size of similar studies published in literature [1, 5].

4.2.2.4 Study endpoints

The primary endpoint was the association of Cat-G levels with disease severity, secondary endpoints were the association of Cat-G levels with bronchiectasis patients' characteristics (demographics, aetiology and clinical status), with both standard microbiology and microbiome and the comparison of aNE and Cat-G characteristics in predicting severe BSI, microbiology and severe exacerbations.

4.2.3 Results

4.2.3.1 Study population and clinical analysis

320 patients from both Policlinico Hospital (Milano, Italy), Ninewells hospital (Dundee, UK) and Hospital de la Santa Creu i Sant Pau, (Barcelona, Spain) and were enrolled to the study [67.2% (215) female, age 67 (58-74) years]. 245 of these patients were reported to have detectable levels of Cat-G and were included in the study. Patients were divided in three groups based on Cat-G tertiles. Specifically, 82 patients had less than 40 ng/mL Cat-G (Low), 82 between 40 and 117 ng/mL and 81 more than 117 ng/mL in sputum. Comparison between the three study groups is reported in table 1.

Table 1. Clinical characteristics of the study population and according to the three study groups

		Sputum Cathepsin G level			**Study population (n= 245)**	**p-value**
		Low (<40 ng/ml) (n= 82)	**Medium (40-117 ng/ml) (n= 82)**	**High (>117 ng/ml) (n= 81)**		
Demographics						
Centre, n (%)	Dundee	40 (48.8)	7 (8.5)	24 (29.6)	71/245 (29.0)	-
	Milan	18 (22.0)	61 (74.4)	50 (61.7)	129/245 (52.7)	
	Barcelona	24 (29.3)	14 (17.1)	7 (8.6)	45/245 (18.4)	
Males, n (%)		33 (40.2)	20 (24.4)	24 (29.6)	77/245 (31.4)	0.08
Median (IQR) age, years		68 (62-73)	65 (54-73)	67 (59-74)	67 (59-74)	0.47
Age >65 years, n (%)		49 (59.8)	40 (48.8)	46 (56.8)	135/245 (55.1)	0.34
Median (IQR) BMI, kg/m^2		24.8 (22.3-29.5)	23.0 (20-25.0)	22 (19.1-25.3)	23.3 (20.0-26.4	0.0001[(1)]
Underweight (BMI <18.5 kg/m^2), n (%)		3 (3.8)	11 (13.4)	13 (16.1)	27/243 (11.1)	0.03[(2)]
Former or current smoker, n (%)		35 (43.2)	30 (37.0)	26 (32.1)	91/243 (37.5)	0.34
Disease severity						
Median (IQR) mMRC		1 (1-2)	1 (0-2)	1 (0-2)	1 (0-2)	0.03[(3)]
mMRC 3-4, n (%)		12 (14.8)	8 (9.8)	16 (19.8)	36/244 (14.8)	0.20
Median (IQR) Reiff score		2.5 (1-3)	4 (3-8)	4 (3-8)	4 (2-8)	0.0001[(4)]
Reiff significance for BSI, n (%)		29 (50.0)	58 (85.3)	59 (80.8)	146/199 (73.4)	<0.0001[(5)]
Median (IQR) BSI		5 (4-8)	7 (4-9)	8 (5-12)	6 (4-9)	0.008[(6)]
BSI risk class, n (%)	Mild	31 (38.3)	25 (30.9)	17 (21.0)	73/243 (30.0)	0.06
	Moderate	35 (43.2)	32 (39.5)	28 (34.6)	95/243 (39.1)	0.53
	Severe	15 (18.5)	24 (29.6)	36 (44.4)	75 (30.9)	0.002[(7)]

Median (IQR) E-FACED		2 (1-3)	3 (1-4)	3 (1-4)	2 (1-4)	0.01[8]
E-FACED risk class, n (%)	Mild	60 (76.0)	56 (69.1)	48 (59.3)	164/241 (68.1)	0.08
	Moderate	11 (13.9)	24 (29.6)	21 (25.9)	56/241 (23.2)	0.049[9]
	Severe	8 (10.1)	1 (1.2)	12 (14.8)	21 (8.7)	0.004[10]
Aetiology						
Aetiology, n (%)	AAT deficiency	0 (0.0)	0 (0.0)	1 (1.2)	1 (0.4)	0.06
	ABPA	2 (2.4)	1 (1.2)	0 (0.0)	3 (1.2)	
	Aspiration	0 (0.0)	0 (0.0)	2 (2.5)	2 (0.8)	
	Asthma	2 (2.4)	0 (0.0)	2 (2.5)	2 (0.8)	
	CFTR-RD	2 (2.4)	1 (1.2)	1 (1.2)	4 (1.6)	
	COPD	5 (6.1)	1 (1.2)	4 (4.9)	10 (4.1)	
	Connective Tissue Disease	2 (2.4)	1 (1.2)	4 (4.9)	10 (4.1)	
	IBD	3 (3.7)	0 (0.0)	2 (2.5)	5 (2.0)	
	Idiopathic	50 (61.0)	47 (57.3)	42 (51.9)	139 (56.7)	
	Post-infective	12 (14.6)	12 (14.6)	12 (14.8)	36 (14.7)	
	Primary Ciliary Dyskinesia	3 (3.7)	6 (7.3)	5 (6.1)	14 (5.7)	
	Primary Immunodeficiency	3 (3.7)	6 (7.3)	5 (6.2)	14 (5.7)	
	Secondary Immunodeficiency	0 (0.0)	4 (4.9)	0 (0.0)	4 (1.6)	
	Other	1 (1.29)	1 (1.2)	1 (1.2)	3 (1.2)	
Clinical status						
Daily sputum, n (%)		54 (93.1)	68 (100.0)	72 (97.3)	194/200 (97.0)	0.08
Median (IQR) sputum volume		11 (5-259	15 (5-40)	20 (5-50)	15 (5-40)	0.34
Median (IQR) exacerbation previous year		1 (1-3)	2 (1-3)	2 (1-3)	2 (1-3)	0.84
>2 exacerbations/previous year, n (%)		40(48.8)	45(56.3)	48(59.3)	133/243 (54.7)	0.384
>3 exacerbations/previous year, n (%)		30(36.6)	26(32.5)	28(34.6)	84/243(34 .6)	0.861
Median (IQR) hospitalization previous year		0 (0-0)	0 (0-0)	0 (0-1)	0 (0-0)	0.01[11]
>1 hospitalization previous year, n (%)		14(17)	8(10)	23(28)	45/244 (18.4)	0.012[12]
Median (IQR) QoLB questionnaire-Respiration		66.7 (55.6-76.1)	70.4 (55.6-79.2)	55.6 (37.0-74.1)	66.7 (48.1-76.1)	0.008[13]
Standard Microbiology						
Chronic infection, n (%)		26 (31.7)	40 (48.8)	60 (74.1)	126/245 (51.4)	<0.0001[14]
Chronic infection other bacteria, n (%)		20 (24.4)	23 (28.1)	22 (27.2)	65/245 (26.5)	0.86
Chronic *P. aeruginosa* infection, n (%)		9 (11.0)	22 (26.8)	40 (49.4)	71/245 (29.0)	<0.0001[15]
Chronic MSSA infection, n (%)		8 (9.8)	5 (6.1)	6 (7.4)	19/245 (7.8)	0.67
Chronic *H. influenzae*/*parainfluenzae* infection, n (%)		8 (9.8)	11 (13.4)	10 (12.4)	29/245 (11.8)	0.76
Chronic *S. pneumoniae*infection, n (%)		2 (2.4)	3 (3.7)	0 (0.0)	5 (2.0)	0.38
Chronic MRSA infection, n (%)		0 (0.0)	0 (0.0)	1 (1.2)	1/245 (0.4)	0.33
Chronic *Stenotrophomonas* infection, n (%)		2 (2.4)	0 (0.0)	0 (0.0)	2/245 (0.8)	0.14
Chronic *Achromobacter* infection, n (%)		0 (0.0)	1 (1.2)	2 (2.5)	3/245 (1.2)	0.36

Other chronic infection, n (%)	4 (4.9)	7 (8.5)	5 (6.2)	16/245 (6.2)	0.66
Chronic infection A. fumigatus, n (%)	1 (1.2)	0 (0.0)	0 (0.0)	1 (0.4)	0.37

Definition of abbreviations: BSI= Bronchiectasis severity index; MRSA= Methicillin-Resistant Staphylococcus Aureus; MSSA= Methicillin- sensitive Staphylococcus Aureus; COPD = chronic obstructive pulmonary disease;

Data are presented as median (interquartile range) or n (%).

1. Low VS. Medium p-value= 0.0003; Low VS. High p-value= 0.0001.
2. Low VS. Medium p-value= 0.03; Low VS. High p-value= 0.009.
3. Low VS. Medium p-value= 0.02.
4. Low VS. Medium p-value <0.0001; Low VS. High p-value <0.0001.
5. Low VS. Medium p-value <0.0001; Low VS. High p-value <0.0001.
6. Low VS. High p-value= 0.003.
7. Low VS. High p-value= 0.0004.
8. Low VS. High p-value= 0.005.
9. Low VS. Medium p-value= 0.02.
10. Low VS. Medium p-value= 0.01; Medium VS. High p-value= 0.001.
11. Medium VS. High p-value= 0.004.
12. Medium VS. High p-value= 0.004.
13. Medium VS. High p-value= 0.004.
14. Low VS. Medium p-value= 0.03; Low VS. High p-value <0.0001; Medium VS. High p-value= 0.0009.
15. Low VS. Medium p-value= 0.01; Low VS. High p-value <0.0001; Medium VS High= 0.003.

BMI was reported to be different between the three categories of Cat-G, and specifically the rate of underweight was statistically lower in the low group compared to both medium and high [low 3 (3.8) vs medium 11 (13.4) vs high 13 (16.1), p=0.03; Low VS. Medium p-value= 0.03; Low VS. High p-value= 0.009].

Disease severity in terms of BSI was reported to be directly correlated with Cat-G (rho=0.27, p<0.0001) and increased the low Cat-G group in comparison to the high one (Low 5 (4-8), medium 7 (4-9) and high 8 (5-12), p=0.008; Low vs High p= 0.003). Similarly, E-FACED, another disease severity indicator was directly correlated to Cat-G (rho=0.23, p<0.0001) and resulted to be statistically increased in the high category compared to the low one [Low 2 (1-3) vs medium 3 (1-4) vs high 3 (1-4), p= 0.01; Low VS. High p-value= 0.005]. Moreover, Reiff score (radiological severity) resulted to be correlated to Cat-G (rho=0.2, p<0.0001) and increased in the medium and high groups compared to the low one [low 2.5 (1-3) vs medium 4 (3-8), vs high 4 (3-8), p= 0.0001; Low VS. Medium p-value <0.0001; Low VS. High p-value <0.0001].

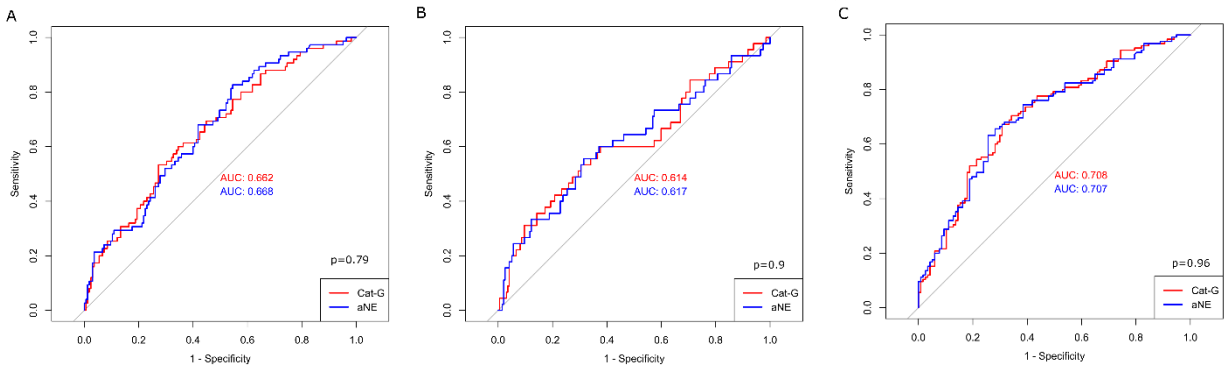
Patients with at least one hospitalized exacerbation in the year before enrolment were increased in the high group compared to both low and medium (Low 14 (17%), VS. medium 8 (10%), VS. high 23(28%), p=0.012, 1. Medium VS. High p-value= 0.004). Finally, respiration section of QoL-B results were lower in the high group compared to both medium and low [Low 66.7 (55.6-76.1), vs medium 70.4 (55.6-79.2), vs high 55.6 (37.0-74.1), p=0.008; Medium VS. High p-value= 0.004].

In terms of microbiology, both patients with medium and high levels of Cat-G resulted to have a higher frequency of chronic infection compared to the ones with low levels. Specifically, chronic *P. aeruginosa* infection resulted to be increasing with the levels of Cat-G [Low 9 (11.0%) vs medium 22 (26.8%) vs high 40 (49.4%), p<0.0001; Low VS. Medium p-value= 0.01; Low VS. High p-value <0.0001; Medium VS High= 0.003].

4.2.3.2 Cat-G and aNE

The comparison of Cat-G and aNE ability in patients outcomes lead to the identification of no statistical difference among ROC curves of the two biomarkers in predicting severe BSI (severe BSI Cat-G AUC=0.662, aNE AUC=0.668, p=0.79), severe exacerbations(hospitalized) (severe BSI Cat-G AUC=0.614, aNE AUC=0.617, p=0.9) and chronic infection (severe BSI Cat-G AUC=0.706, aNE AUC=0.707, p=0.96), see figure 1.

Figure 1. ROC curve of Cathepsin G and aNE in predicting A) severe BSI, B) severe exacerbation and C) chronic infection



4.2.3.3 Microbiome and molecular biology assessment

Patients from the Italian and Spanish centre underwent 16s rRNA gene sequencing for microbiome evaluation and molecular detection of respiratory pathogens. Summary of biological characteristics is reported in Table 2.

Table 2. Biological characteristics of the study population according to the three study groups

	Low (<40 ng/ml) (n=18)	**Medium (40-117ng/ml) (n=51)**	**High (>117ng/ml) (n=59)**	**p-value**
Alpha diversity				
Median (IQR) Richness	36 (24-40)	31 (22.5-37)	28.5 (20.5-35)	0.02 (1)
Median (IQR) Evenness	0.7 (0.5-0.8)	0.5 (0.3-0.7)	0.5 (0.3-0.7)	0.001 (2)
Median (IQR) Shannon	2.5 (1.6-2.8)	1.9 (0.9-2.4)	1.5 (1-2.4)	0.002 (3)
Median (IQR) Berger-Parker dominance	0.3 (0.2-0.6)	0.5 (0.3-0.8)	0.6 (0.3-0.8)	0.002 (4)
Molecular detection of respiratory pathogens				
P. aeruginosa detection, n (%)	3 (16.7)	28 (47.5)	31 (60.8)	0.005 (5)
Median (IQR) *P. aeruginosa* genome copies/mL, log	6.7 (5.4-7.3)	7.5 (6.8-8.0)	8.0 (7.5-8.3)	0.01 (6)
H. influenzae detection, n (%)	7 (38.9)	17 (28.8)	10 (19.6)	0.24
Median (IQR) *H. influenzae* genome copies/mL, log	5.4 (4.4-6.7)	7.9 (7.4-9.1)	7.2 (4.5-8.9)	0.09
S. aureus detection, n (%)	8 (44.4)	21 (35.6)	20 (39.2)	0.78
Median (IQR) *S. aureus* genome copies/mL, log	6.7 (5.5-7.3)	4.3 (3.4-7.8)	5.1 (3.8-7.7)	0.56
S. pneumoniae detection, n (%)	8 (44.4)	24 (40.7)	15 (29.4)	0.36
Median (IQR) *S. pneumoniae* genome copies/mL, log	3.6 (3.3-4.5)	3.6 (3.1-5.6)	4.4 (3.1-4.7)	0.86

1. Low VS. High p-value=0.02
2. Low VS. High p-value=0.002
3. Low VS. High p-value=0.002
4. Low VS. High p-value=0.003; Low VS. Medium p-value=0.005
5. Low VS. Medium p-value=0.02; Low vs. High p-value=0.02
6. Medium VS.High p-value=0.04

4.2.3.4 Microbiome analysis

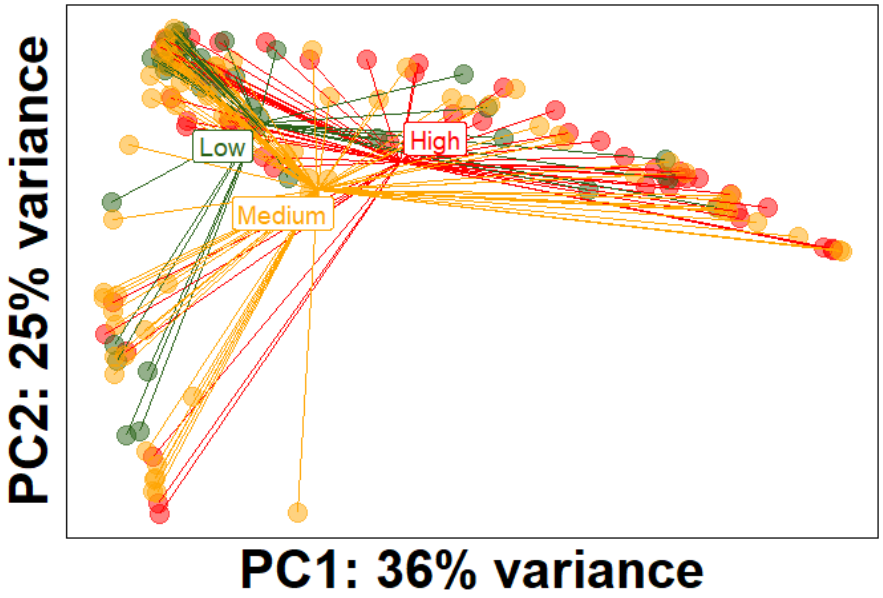
170 patients from the Italian and Spanish cohort with detectable Cat-G levels underwent 16s rRNA gene sequencing for microbiome evaluation. All analysis were performed at genus level.

The analysis of within-sample diversity (alpha) between the three study groups, lead to the detection of a decrease in alpha diversity in terms of Shannon and evenness in the higher groups compared to the low one [Shannon: Low, 2.5 (1.6-2.8) vs. medium 1.9 (0.9-2.4) vs. high 1.5 (1-2.4), p-value=0.002; Low vs High, p=0.002. Richness: Low, 36 (24-40) vs. medium 31 (22.5-37) vs. high 28.5 (20.5-35), p-value=0.02; Low vs. High p-value=0.02 Evenness: Low, 0.7 (0.5-0.8) vs. medium 0.5 (0.3-0.7) vs. high 0.5 (0.3-0.7), p-value=0.002, Low vs. High p-value=0.002]

Berger-Parker dominance index was reported to be increased in both patients with medium and high levels of Cat-G compare to the low ones [Low, 0.3 (0.2-0.6) vs. medium 0.5 (0.3-0.8) vs. high 0.6 (0.3-0.8), p-value=0.002; Low vs. High, p=0.003, Low vs. High p-value=0.003; Low vs. Medium p-value=0.005], even if none of the genera was reported as more frequently dominant than the others (Table 2).

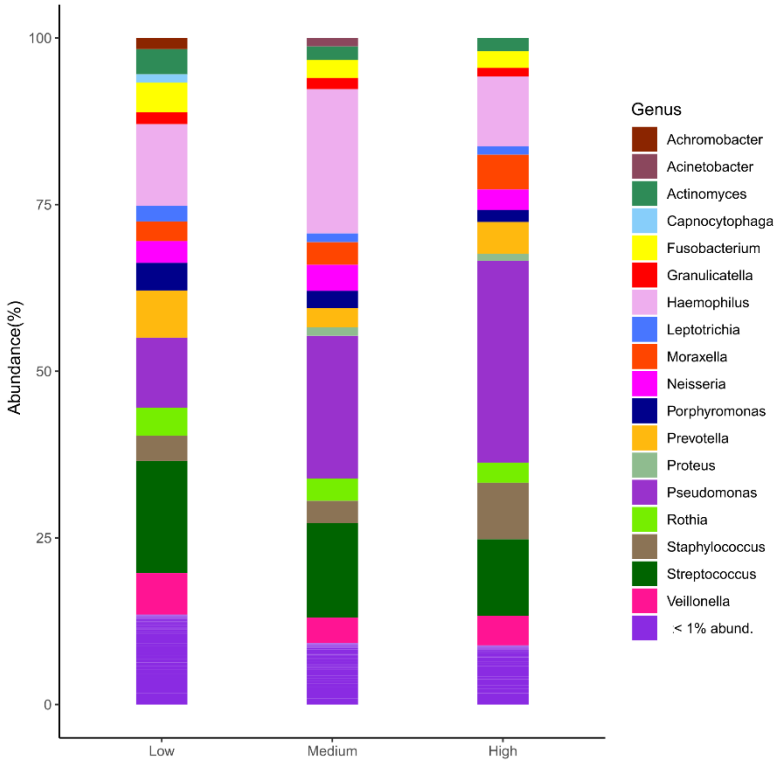
PCoA based on Bray-Curtis dissimilarity showed a clustering between patients with similar levels of Cat-G (PERMANOVA, p=0.001), Figure 2. Abundance barplots at genus level are reported in Figure 2.

Figure 2. PCoA based on Bray-Curtis dissimilarity according to the three study groups



Abundance barplots at genus level are reported in figure 3.

Figure 3. Taxa barplots of relative abundance of genera according to the three study groups



4.2.3.5 Molecular evaluation of respiratory pathogens

Molecular evaluation for the evaluation of respiratory pathogens reported an increased frequency of detection of *P. aeruginosa* between both medium and high groups compared to the low one [Low 3 (16.7%) vs. Medium 28 (47.5%) vs. High 31 (60.8%) p-value=0.005]. Moreover, the analysis of quantification data of *P. aeruginosa* showed an increased level of *P. aeruginosa* genome copies/mL log in the high group compared to the medium one [Low 6.7 (5.4-7.3) Loggenome copies/mL, vs medium 7.5 (6.8-8.0) Log genome copies/mL vs high 8.0 (7.5-8.3) Log genome copies/mL, p=0.01; Medium vs. High p-value=0.04].

Finally, no significant difference emerged from the analysis on *H. influenza*e, *S. pneumoniae* and *S. aureus.*

4.2.4 Discussion

The major findings of our study were: 1) Cat-G levels correlated with microbiome diversity and differences in the detection and quantification on *P. aeruginosa*, 2) Cat-G levels correlated with disease severity, radiological severity, quality of life and chronic infection, 3) No difference was found among Cat-G and aNE in predicting severe BSI, severe exacerbation and chronic infection.

As a new finding, we discovered that Cat-G levels correlated with microbiome diversity and differences in the detection and quantification on *P. aeruginosa*. Microbiome diversity is strictly correlated to disease severity in chronic respiratory diseases and these data may suggest that a dysbiosis contributes to perpetrating disease *status* by alimenting the cycle of inflammation, pulmonary damage and infection. Moreover, experiences in literature on cystic fibrosis reported a decreased pulmonary function in patients with increased load of gram-negatives in sputum, contributing to the idea that an increased burden of these bacteria may be involved in serine proteases increase and parallel increase of disease severity [6]. Disease severity role in potentially increasing dysbiosis is well known in literature and the increase of presence of pathogenic species in patients' lungs may further contribute to this picture of infection inflammation. According to our data, Cat-G seems to contribute to inflammatory status in lungs, as well as neutrophil elastase and to be involved in Cole's cycles of infection, inflammation and tissue damage.

Cat-G also is associated with disease severity in terms of BSI and E-FACED, radiological severity in terms of Reiff score, quality of life (respiratory symptoms) and chronic infection. All these variables are consistent with an increase of disease severity in this disease. Cat-G in bronchiectasis was identified and targeted for inhibition through compounds able to impair cathepsin C activity, however never considered a biomarker for patients' stratification itself. The role of Cat-G is less clear compared to neutrophil elastase. So far, the scientific community is aware of the role of Cat-G in activating airway epithelial cells, increasing mucous secretions, regulating innate immunity and inflammation, and being active against pathogens, especially *P. aeruginosa* [7, 8]. It is also believed that Cat-G may contribute to the pathogenesis of chronic respiratory diseases, being responsible for a decreased *P. aeruginosa* clearance in mice [3]. Cat-G is part of inflammatory response to noxious stimuli in lungs and as a serine protease we can speculate that Cat-G may contribute in the increase of radiological severity directly degrading extracellular matrix or through the activation of matrix metalloproteinasis [7, 8].

Finally, we considered Cat-G and aNE relation and aimed at comparing these two biomarkers performance in predicting disease severity. Even if aNE is a well-studied and known biomarker for bronchiectasis, Cat-G was able to compete with aNE in the prediction of disease severity.

aNE data on predicting disease severity exacerbations are consistent with previously published data on aNE reported by Chalmers's and Gramegna's experiences in respectively northern and southern Europe [9, 10].

This study has some limitations. We included only three European cohorts that were referral centres for bronchiectasis. The inclusion of a larger number of centres may increase the generalizability of the data hereby presented. Moreover, the number of patients may limit the statistical power of our study. This is an exploratory study and no preliminary data were present in literature on this biomarker in bronchiectasis.

Biological samples were evaluated during clinical stability, not considering biomarker stability through time, possible modifications during exacerbations and the effect of therapy.

Patients with undetectable Cat-G were excluded from the study. Being the first study in this field and the lack of a gold standard in the evaluation of Cat-G in sputum, we were not able to define if undetectable data were very low in Cat-G or the kit was failing in detecting Cat-G in samples. An extensive test of this methodology will help researchers to interpret undetectable data. Sputum is not fully representative of low airways, but is easy-accessible and potentially reproducible in clinical practice. Microbiome and molecular biology evaluations were conducted on a smaller cohort including Italian patients.

On the other end, the study design was prospective and multicentrical. It was a translational study considering a great number of clinical and biological variables and the evaluation of clinical and biological variables was consistent across the three centres.

All results are concordant in describing patients with high Cat-G levels with poorer quality of life, higher clinical severity, higher chronic infection and dysbiosis.

The identification of Cat-G as new biomarkers for patients' stratification and outcome prediction in bronchiectasis may have an impact on both clinical and research communities. The development of new tools is going towards the endotyping of a neutrophilic inflammation phenotype, and a new personalized approach in patients care.

In conclusion, Cat-G correlated with clinical and radiological severity, worse quality of life and *P. aeruginosa* chronic infection. Moreover, in a subgroup of patients we described the presence of dysbiosis increasing with Cat-G levels and molecular detection of higher levels of *P. aeruginosa*. Furthermore, Cat-G revealed to be comparable to aNE in predicting disease severity, severe hospitalizations and chronic infection.

4.2.5 References

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Chapter 5

5 Sputum microbiome, inflammation and clinical phenotypes in bronchiectasis

5.1 Microbiome-inflammation network in frequent vs- non-frequent exacerbators with chronic *P. aeruginosa* in bronchiectasis

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5.1.1 Introduction

Bronchiectasis is a chronic respiratory lung disease characterized by an abnormal and permanent dilatation of the bronchi with daily symptoms of cough, sputum production, and frequent respiratory infections [1]. Exacerbations are clinically important in bronchiectasis and may lead to disease progression in terms of inflammation, lung function impairment and mortality. The frequency of exacerbation represents a meaningful outcome in bronchiectasis and is used both in clinical practice and in research to monitor patients' disease status [2].

Chronic *Pseudomonas aeruginosa* airway infection is a well-known risk factor for mortality, hospitalizations and exacerbations in bronchiectasis [3, 4]. In clinical practice, we recognize bronchiectasis patients with chronic *P. aeruginosa* infection who exhibit a frequent or non-frequent exacerbator phenotype. Araujo and colleagues reported that among patients with chronic *P. aeruginosa* infectionthose with two or more exacerbations per year have a higher mortality rate than those with fewer than two exacerbations per year [5]. The reason why bronchiectasis patients with chronic *Pseudomonas* infection might be either frequent or non-frequent exacerbators is still unknown. Possible differences might reside in the host-pathogen interaction, including the role of *Pseudomonas* within the airway microbiota and related inflammatory patterns. In order to have a deeply investigate host-pathogen interaction, researchers are nowadays exploring interaction analysis among microbial community and host response [6]. Few studies have been published so far in respiratory diseases addressing host-pathogen interaction as a network, to identify patients' characteristics non-detectable through single analysis [7, 8]. We, thus hypothesize that the difference among frequent and non-frequent exacerbators may reside in the interaction of *Pseudomonas* with the other bacterial genera and in the host-pathogen interaction evaluated through network analysis.

5.1.2 Materials and Methods

5.1.2.1 Study design and population

An observational, cross-sectional study was performed at the Bronchiectasis Program of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, between March 2017 and March 2019. Adults (≥ 18 years) with clinically (daily sputum production) and radiologically significant bronchiectasis with chronic *P. aeruginosa* infection were enrolled during stable state. Chronic *P. aeruginosa* infection was defined by the isolation of *P. aeruginosa* in sputum culture on two or more occasions, at least 3 months apart over the 1-year period before the enrolment [14]. In addition, as an inclusion criteria, sputum at enrolment should have been positive for *P. aeruginosa* through Real-time PCR. Patients with either cystic fibrosis (CF) or traction bronchiectasis due to pulmonary fibrosis were excluded. The study was approved by the local ethical committee, and all subjects provided a written informed consent.

5.1.2.2 Study procedures

Bronchiectasis patients underwent clinical, radiological, microbiological and functional evaluation (see "Supplementary material" of chapter "Sputum Neutrophil Elastase associates with microbiota and *P. aeruginosa* in bronchiectasis" of this thesis). Spontaneous sputum samples were obtained and mucous plugs were isolated. DNA extraction, as well as sputum supernatant, bacterial Real-Time PCR and microbiome evaluation (and analysis) were carried out following the procedures described in "Supplementary material" of chapter "Sputum Neutrophil Elastase associates with microbiota and in *P. aeruginosa* bronchiectasis" of this thesis.

5.1.2.3 Clinical variables and study definitions

Demographics, comorbidities, disease severity, etiology of bronchiectasis, respiratory symptoms, sputum evaluation, radiological, functional and biological characteristics in stable state were recorded. Disease severity was evaluated according to both the Bronchiectasis Severity Index (BSI) and FACED score (evaluating FEV_1, Age, Chronic infection with *Pseudomonas*, Radiological Extension and Dyspnea). [9, 10]. Radiological severity of bronchiectasis was assessed using a modified Reiff score, which rates the number of involved lobes (with the lingula considered to be a separate lobe) and the degree of dilatation (range: 1-18). [11] All bacteriology was performed on spontaneous sputum samples as previously described. [12] Murray-Washington criteria for sputum quality was used in all cases, with all samples having less than 10 squamous cells and more than 25 leukocytes per low-power microscope field. Chronic

infection was definedby the isolation of potentially pathogenic bacteria in sputum culture on two or more occasions, at least 3 months apart over a 1-year period. [13]

5.1.2.4 Study groups

Patients were divided in two groups based on the number of exacerbations they experienced in the year prior the enrolment according to Arujo et al.: 0-1 exacerbations/year (non-frequent exacerbators) vs. 2+ exacerbations/year (frequent exacerbators) [5].

5.1.2.5 Statistical analysis

Variables were collected in an *ad hoc* electronic form. Qualitative variables were summarized with absolute and relative (percentage) frequencies, whereas quantitative variables with medians (interquartile ranges, IQR). Qualitative variables were statistically assessed with chi-squared or Fisher exact test, when appropriate, whereas those for quantitative non-parametric variables with Mann-Whitney. Continuous variables were assessed with Spearman correlation. A two-tailed p-value was considered statistically significant when less than 0.05. The statistical software SPSS version 25 (IBM, Armonk, USA) was used for all statistical computations.

5.1.2.6 Association network analysis

Association networks were generated using a generalized boosted linear models (GBLMs) as described by Faust *et al.*[14]. Normalised cytokine and microbiome data were combined as input to infer the maximal cytokine-microbial associations.

5.1.3 Results

5.1.3.1 Study population

Among the 47 patients [70.2% female; median (IQR) age: 63 (53-71) years] enrolled, 15 (31.9%) were non-frequent and 32 (68.1%) were frequent exacerbators. Characteristics of the two study groups are depicted in Table 1. The only differences found among the two groups were an increased number of patients with more than 65 years and increased incidence of Gastro-oesophageal reflux disease in the frequent exacerbators group.

Table 1. Clinical characteristics of the study population according to the two study groups

Demographics		**Non-frequent exacerbators (N=15)**	**Frequent exacerbators (N=32)**	**p-value**
Female, n (%)		11 (73.3%)	22 (68.8%)	1
Median (IQR) age, years		64 (54-73)	66 (55-71)	0.123
Age >65, n (%)		3 (20%)	18 (56.3%)	0.028
Median (IQR) BMI		20 (19-24)	21 (18.8-24)	0.373
Former/current smokers, n (%)		6 (40%)	13 (40.6%)	0.968
Comorbidities (most frequent)				
Gastro-oesophageal reflux disease, n (%)		3 (20%)	18(56.3%)	0.028
Cardiovascular disease, n (%)		2(13.3%)	14(43.8%)	0.052
Rhinosinusitis, n (%)		7(46.7%)	8(25%)	0.137
Osteoporosis, n (%)		2(13.3%)	6(18.8%)	0.645
Nasal polyps, n (%)		3(20%)	4(12.5%)	0.664
Depression, n (%)		2(13.3%)	3(9.4%)	0.648
Anxiety, n (%)		3(20%)	2(6.3%)	0.309
Radiology				
Median (IQR) Reiff score		6 (4-8)	6 (3.25-12)	0.982
Disease severity				
Median (IQR) BSI		9 (7-10)	9 (6-13)	0.195
BSI risk classes, n (%)	Mild	0 (0%)	1 (3.1%)	1
	Moderate	7 (46.7%)	9 (28.1%)	0.211
	Severe	8 (53.3%)	22 (68.8%)	0.305
Median (IQR) FACED		3 (2-5)	3 (3-5)	0.133
FACED risk classes, n (%)	Mild	6 (40%)	8 (25%)	0.295
	Moderate	7 (46.7%)	13 (40.6%)	0.696
	Severe	2 (13.3%)	11 (34.4%)	0.175
Aetiology				
Idiopathic, n (%)		8 (53.3%)	21 (65.6%)	0.419
Immunodeficiency, n (%)		2 (13.4%)	4 (12.5%)	0.936

Primary Ciliary Dyskinesia, n (%)		4 (26.6%)	2 (6.3%)	0.072
Post infective, n (%)		0 (0%)	2 (6.3%)	1
Others*, n (%)		1 (6.7%)	3 (9.3%)	0.756
Clinical Status				
Sputum colour, n (%)	Mucous	2 (14.3%)	2 (6.7%)	0.581
	Mucopurulent	6 (42.9%)	11 (36.7%)	0.694
	Purulent	6 (42.9%)	17 (56.7%)	0.521
Median (IQR) sputum volume (mL)		30 (10-100)	25 (5-75)	0.886
Inhaled antibiotics, n (%)		1 (6.7%)	2 (6.3%)	0.957
Azithromycin, n (%)		0 (0%)	5 (15.6%)	0.162
Lung function				
Median (IQR) FEV_1 %		81 (70-96)	68 (52-84)	0.297
Chronic infection (other than *P. aeruginosa)*				
H. influenzae, n (%)		2 (13.3%)	0 (0%)	0.097
S. aureus, n (%)		1 (6.7%)	2 (6.7%)	1
S. maltophilia, n (%)		1 (6.7%)	1 (3.1%)	0.575
A. fumigatus, n (%)		1 (6.7%)	0 (0%)	0.319
E. coli, n (%)		0 (0%)	1 (3.1%)	1
Molecular biology				
Median (IQR) *P. aeruginosa* genome copies		1.03E+08 (34,660,664.82-139,889,196.68)	112707756.23 (10,249,307.48-210,353,185.6)	0.802
H. influenzae detection, n (%)		1 (6.7%)	9 (28.1%)	0.135
Median (IQR) *H. influenzae* genome copies		1,576,923 (1,576,923.08-1,576,923.08)	42,564.1 (2,1923.08-137,179.49)	0.4
S. aureus detection, n (%)		5 (33.3%)	11 (34.4%)	0.944
Median (IQR) *S. aureus* genome copies		4,460.13 (1,918.6-12,790.7)	2,134.55 (773.26-8,629.57)	0.441
S. pneumoniae detection, n (%)		6 (40%)	7 (21.9%)	0.195
Median (IQR) *S. pneumoniae* genome copies		19,521.59 (589.2-46,392.05)	1,954.55 (953.41-3,704.55)	0.836
Alpha diversity				
Median (IQR) Richness		46 (16-76)	49.5 (29-69)	0.802
Median (IQR) Evenness		0.66 (0.54-0.813)	0.69 (0.5-0.82)	0.891
Median (IQR) Shannon		2.95 (2.14-5.08)	3.68 (2.6-5)	0.837
Inflammatory biomarkers				
Median (IQR) active neutrophil elastase, µg/ml		39.97 (17.45-54.04)	34.34 (21.36-47.01)	0.451
Median (IQR) TNFα, pg/mL		1,422.2 (967.02-1,547.86)	1,158.43(942.15-1,471.48)	0.307
Median (IQR) IL6, pg/mL		227.16 (164.45-278.03)	146.58(119.92-286.39)	0.525
Median (IQR) IL1β, pg/mL		483,166.83 (265,602.62-705,337.84)	266,147.03 (187,109.59-590,444.98)	0.094
Median (IQR) IL10 pg/mL		31,590.92 (10,084.22-86,131.26)	18,175.1(7,252.64-31,954.66)	0.026

*Other aetiologies include alpha1-antitrypsin deficiency, asthma, non-tuberculous mycobacteria infection and COPD

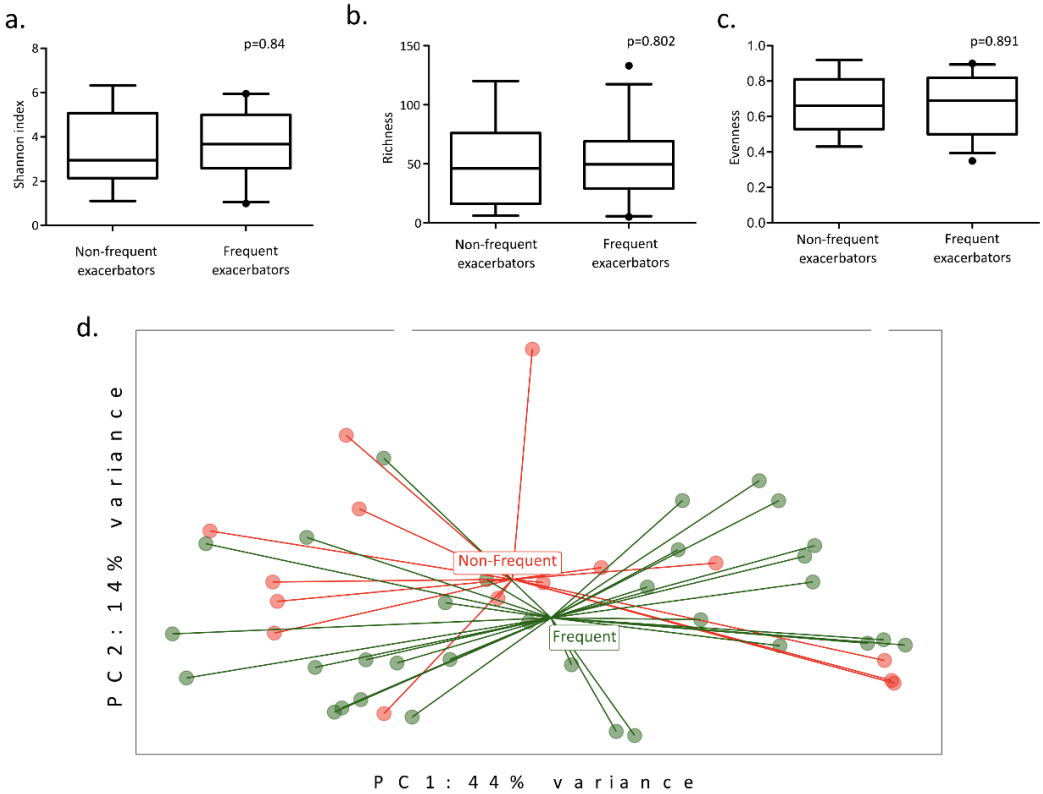
Definition of abbreviations: BSI= Bronchiectasis severity index; COPD = chronic obstructive pulmonary disease.

Data are presented as median (interquartile range) or n (%).

5.1.3.2 Microbiota evaluation

Within-sample diversity was similar in the two study groups in terms of Shannon index, richness and evenness, data are reported in Figure 1a-c and Table 1.Neither Shannon index (rho=0.054; p=0.72), nor richness (rho=-0.059, p=0.69), nor evenness (rho=-0.078, p=0.60) correlated with the number of exacerbations. Beta diversity evaluated as Bray-Curtis dissimilarity did not differ among the two study groups see figure 1d. No enrichment for specific genera was found through DESeq evaluation.

Figure 1. Comparison of median levels of Shannon index (a), richness (b), evenness (c), PCoA of genera based on Bray-Curtis distance (d)



5.1.3.3 Microbial association network analysis of Pseudomonas

A different microbial association of *Pseudomonas* was documented at thebacterialnetwork analysis in frequent vs non-frequent exacerbators, see Figure 2. Strength of the associations is reported in table 2. All associations shown between *Pseudomonas* and other bacteria were inverse. In non-frequent exacerbators*Pseudomonas* was associated with *Peptostreptococcus*, while in frequent exacerbators it was associated with *Moryella*, *Neisseria*, *Streptococcus*, *Fusobacterium*, *Moraxella*, *Oribacterium* and *Aggregabacter*. *Pseudomonas* was associated with *Prevotella*, *Rothia*, *Haemophilus* and *Campylobacter* in both study groups.

Figure 2. Microbial association network of *Pseudomonas* showing statistically significant (p-value <0.001) relationships between microbes in frequent (dotted lines) and non-frequent exacerbators (dashed lines), shared associations are represented by solid lines

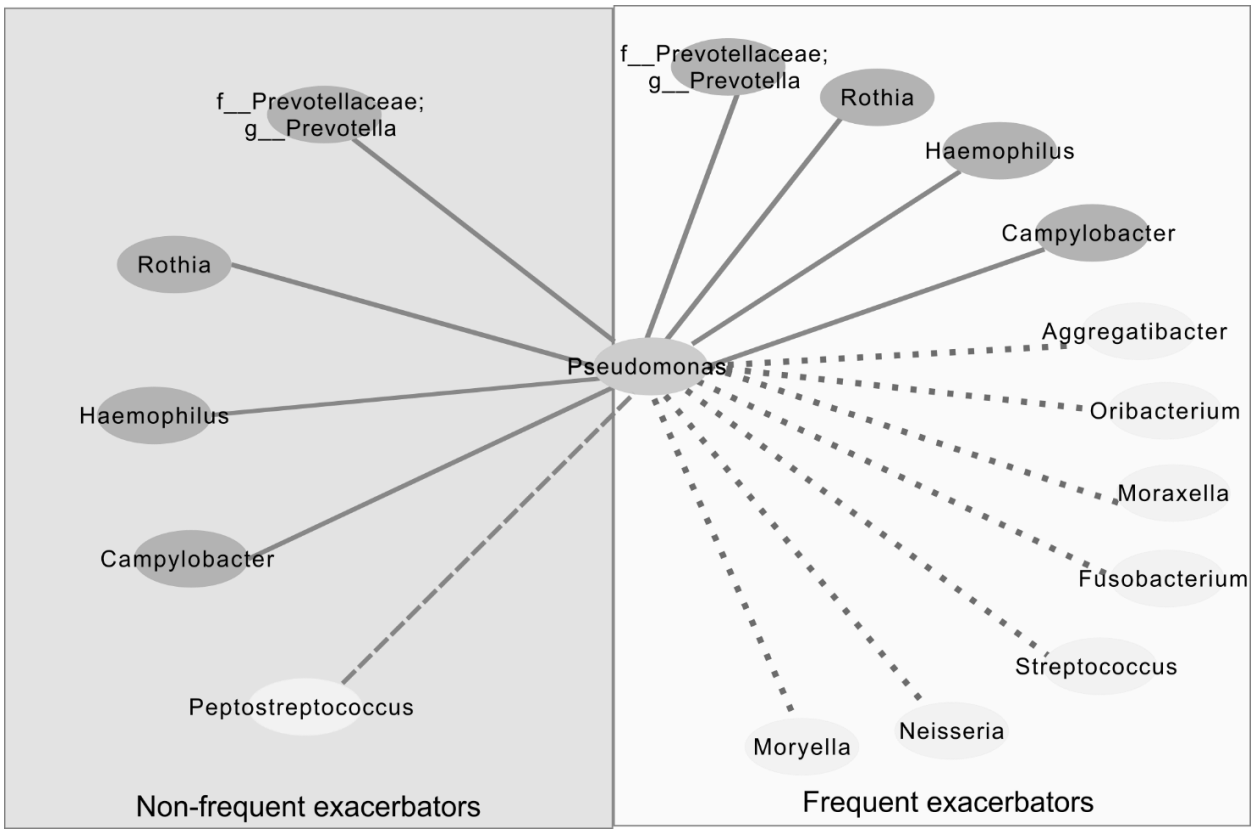


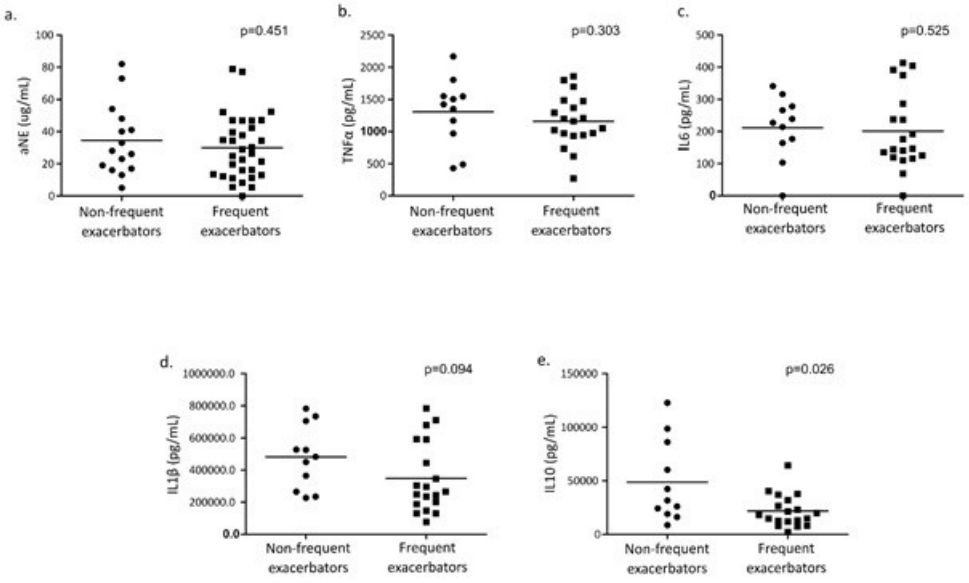
Table 2. Strength of the associations between *Pseudomonas* and other microbes (coupled with figure) derived using a GBLM model. Only statistically significant associations (p-value < 0.001) are reported (f_: family, g_: genus).

Non-frequent exacerbators		
		weight
Pseudomonas	*f__Prevotellaceae, g__Prevotella*	*-1.8935*
Pseudomonas	*Campylobacter*	*-8.7*
Pseudomonas	*Haemophilus*	*-0.8519*
Pseudomonas	*Peptostreptococcus*	*-0.2452*
Pseudomonas	*Rothia*	*-5.7308*
Frequent exacerbators		
Pseudomonas	*Rothia*	*-0.8005*
Pseudomonas	*Fusobacterium*	*-0.4786*
Pseudomonas	*f__Prevotellaceae; g__Prevotella*	*-0.7513*
Pseudomonas	*Neisseria*	*-1.0743*
Pseudomonas	*Campylobacter*	*-2.5758*
Pseudomonas	*Aggregatibacter*	*-0.5557*
Pseudomonas	*Haemophilus*	*-0.2871*
Pseudomonas	*Moraxella*	*-0.7432*
Pseudomonas	*Streptococcus*	*-0.8606*
Pseudomonas	*Moryella*	*-4.2613*
Pseudomonas	*Oribacterium*	*-2.4292*

5.1.3.4 Sputum inflammation

In terms of airway inflammation, levels of pro-inflammatory cytokines (IL1β, IL6 and TNFα) and aNE in sputum were similar among the two study groups. No correlation between exacerbation rate and aNE was found (r=0.032; p=0.83). Significantly lower levels of IL10 were detected in bronchiectasis patients with chronic *P. aeruginosa* infection who were frequent exacerbators [18,175 (7,253-31,955) pg/mL] vs. non-frequent exacerbators [31,591 (10,084-86,131) pg/mL], p=0.026. Levels of IL10 were also inversely correlated with the number of exacerbation (rho=-0.41, p=0.024), see figure 3.

Figure 3. Comparison of median levels of aNE (a), TNFα (b), IL6 (c), IL1β (d) and IL10 among frequent and non-frequent exacerbators



5.1.3.5 Inflammation association network analysis

In non-frequent exacerbators, network analysis at inflammation level showed an implication of twelve bacteria in the inflammation network. Specifically, TNFα expression was directly associated to *Tannerella*, *Prevotella* (Prevotellaceae), while inversely associated with *Aggregatibacter*, *Moraxella*, *Pseudomonas*, and *Neisseriaceae* (as family). IL6 was inversely associated with *Prevotella* (*Paraprevotellaceae*), *Peptostreptococcus* and *Gemellaceae* (family). IL10 expression was associated with Leptotrichia. aNE was associated with *Aggregatibacter*, *Moraxella* and Neisseriaceae (family). Concerning association between inflammatory biomarkers IL6 was inversely associated with TNFα and IL10 with IL1β, see figure 4a.

In frequent exacerbators, network showed a broader association between cytokines and involved a reduced number (4) of bacteria. TNFα was directly associated with *Parvimonas*, while inversely associated to *Haemophilus* and *Fusobacterium*. IL10 expression was directly associated with *Staphylococcus*. Concerning association between cytokines, TNFα was directly associated with aNE, IL6 was inversely associated with IL10, IL1β and aNE and IL10 expression was inversely associated with IL1β and IL6, see figure 4b. Specific data on the association of between cytokines and microbiota are shown in table 3.

Figure 4. Inflammatory association network showing statistically significant associations (p-value <0.001) between inflammatory biomarkers and microbes in non-frequent (a) and frequent exacerbators (b)

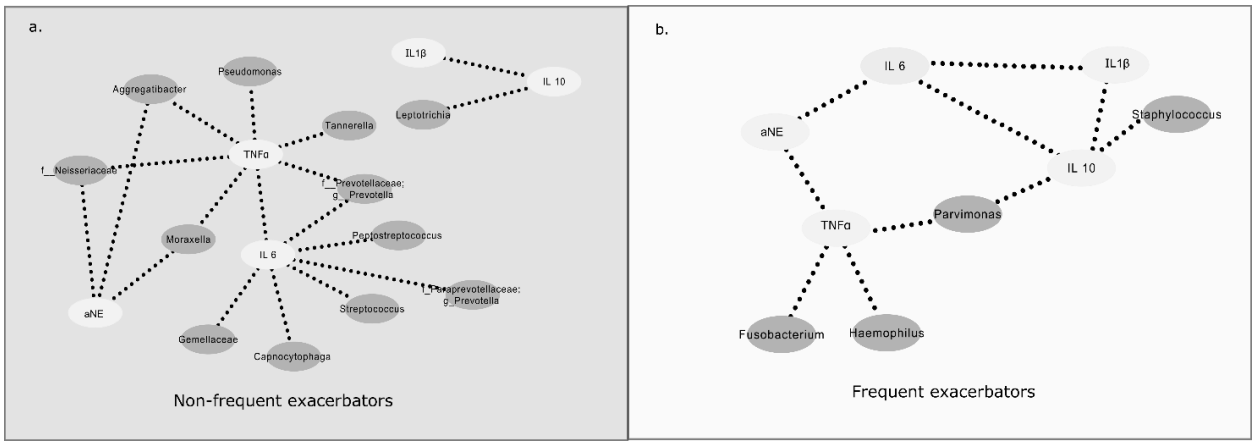


Table 3. Strength of the associations between microbes and inflammatory biomarkers (coupled with figure) derived using a GBLM model. Only statistically significant associations (p-value < 0.001) are reported (f_: family, g_:genus).

Non-frequent exacerbators		
		weight
TNFα	*Aggregatibacter*	-0,0078
Aggregatibacter	aNE	-34,2422
f__*Neisseriaceae*	aNE	-21,1074
Moraxella	aNE	-1,2619
IL6	f__*Gemellaceae*	-0,0034
TNFα	f__*Neisseriaceae*	-0,0279
IL6	f__*Prevotellaceae*; g__*Prevotella*	0,0003
TNFα	f__*Prevotellaceae*; g__*Prevotella*	0,0091
IL6	f_*Paraprevotellaceae*; g_*Prevotella*	-0,0002
IL1β	IL10	-0,7945
Leptotrichia	IL10	-0,0043
Capnocytophaga	IL6	-0,393
Peptostreptococcus	IL6	-1,0714
TNFα	IL6	2,7521
TNFα	*Moraxella*	-0,0747
TNFα	*Pseudomonas*	-0,0004
IL6	*Streptococcus*	0,0003
TNFα	*Tannerella*	0,0137
	Frequent exacerbators	
TNFα	*Haemophilus*	-0,0087
IL1β	IL10	-0,9923
Parvimonas	IL10	-0,1668
IL10	IL6	-0,0357
IL1β	IL6	-1,4103
IL6	aNE	4,0333
TNFα	aNE	11,5442
TNFα	*Parvimonas*	0,0115
IL10	*Staphylococcus*	0,0087
Fusobacterium	TNFα	-0,7202

5.1.4 Discussion

The most important findings of this study are: 1) *Pseudomonas* was associated through network analysis with a higher number of genera in frequent exacerbators compared to non-frequent ones; 2) Inflammation network analysis in frequent exacerbators was associated with a lower number of bacteria and cytokines were directly related to each other; 3) IL10 was reduced in frequent exacerbators; 4) No differences in terms of microbiota diversity and composition were found among the two groups.

Chronic *P. aeruginosa* infection is a well-known predictor for negative outcomes and used in clinical practice to evaluate patient's disease status in bronchiectasis. Network association of *Pseudomonas* with other genera allowed us to focus on its interactions inside the microbiota. This bacterium installed negative associations with all the other bacteria in the network, confirming studies reporting *Pseudomonas* to be a direct and indirect inhibitor of other bacterial species as *H. influenzae* [15]. Moreover, the increased number interactions of *Pseudomonas* with other bacteria suggest us a higher influence of *Pseudomonas* in shaping frequent exacerbators microbiome.

Inflammation network and inflammatory biomarkers levels comparison in the two groups was aimed at investigating host-pathogen interaction. While in non-frequent exacerbators an interplay among cytokines involving 12 bacterial genera frequently mediating between inflammatory molecules was observed, frequent exacerbators network was mainly composed of cytokines directly interacting with each other, with a reduced number of bacteria associated. Interestingly, none of the two networks showed *Pseudomonas* directly interacting with aNE, a well know inflammatory biomarker associated with *Pseudomonas* presence and load. The relation between aNE and *Pseudomonas* seems to be indirect [16].

Moreover, IL10 was found to be lower in frequent exacerbators compared to non-frequent. IL10 is often reported as a pro-resolving factor through the modulation of the expression of different pro-inflammatory cytokines from monocytes, lymphocytes, neutrophils and eosinophils [17]. Low levels of IL10 have been previously detected in patients with asthma, COPD, and CF when compared to healthy population [18–20]. Focusing of the association of IL10 in the two cytokines network, it interacts with IL1β and *Leptotrichia* genus in non-frequent exacerbators, while is included in the large inflammatory association network in frequent exacerbators and directly linked to the expression of both IL1β and IL6 that both have a role as pro-inflammatory cytokines. IL10 dysregulation and its network may suggest a perturbation in pro/anti-

inflammatory effectors balance that should contribute to the increase in frequency of exacerbations.

The lack of differences in microbiota in terms of alpha, beta diversity and enrichment analysis suggest us that the analysis of microbiota composition *per se* is not enough to understand differences among frequent and non-frequent bronchiectasis patients with chronic *P. aeruginosa* infection.

The *primo movens* of this experience was the clinical need of understanding the reason behind the condition of frequent and non-frequent exacerbators in *P. aeruginosa* chronically infected patients with bronchiectasis that were reported by Araujo *et al.* to show differences in long term outcomes. Network association analysis of *Pseudomonas* and of cytokines with microbiome allowed us to face this topic with a more comprehensive approach. Our analysis seems to suggest that the evaluation of single variables was not able to characterize the two study groups, while a complex approach able to consider interactions and interplay of all effectors succeeded in differentiating from a molecular point of view frequent by non-frequent exacerbators. The analysis of microbiome and inflammation allowed us to understand that the peculiarity of frequent exacerbators are the interaction of *Pseudomonas* with a larger group of bacteria in an environment of a more entangled inflammation network. From these data, we may speculate that in frequent exacerbators' lungs, the interaction of *Pseudomonas* with a large number of bacteria in an environment of direct interplay of cytokines that seems able to self-sustain leads to a decrease of IL10, an anti-inflammatory cytokine that have a role in regulating airway inflammation. These data may represent a dysregulated pro/anti-inflammatory biomarkers balance that may be involved in the status of frequent exacerbators of these patients.

Our study is limited by the single centre and cross-sectional sampling. We recognize that sputum sample is not fully representative for lower airways and might show contamination from higher airways. Finally, we did not study several other potential components of the frequent exacerbator *status* in these patients, including the contribution of viral or fungal backgrounds or other potential triggers of exacerbation such as air pollution. Our analysis is the first one of its type published in literature so far and opens a new branch of translational research in bronchiectasis which might give an insight in the next future to a clinically relevant question on the liaison between chronic *P. aeruginosa* infection and exacerbations in bronchiectasis. In conclusion, this experience supports that exacerbation patterns among bronchiectasis patients chronically infected by *P. aeruginosa* is associated with the balance in the pro/anti-inflammatory cytokines ration and network, and on the interactions of *Pseudomonas* with other bacteria rather than

differences in the bacterial community composition and may contribute to understand the increased frequency of exacerbations in these patients.

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Chapter 6

6 General discussion and conclusions

6.1 Main results of this project

This project focused on the evaluation of airway microbiome and inflammation, along with clinical characteristics, of adults with bronchiectasis patients enrolled during stable state at a specialist centres of care for bronchiectasis, mostly the Bronchiectasis Program of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milano, Italy (although some European data are also shown). In details:

1) In the first section of this thesis, we underlined the need to homogenize the methodology used for microbiome evaluation and active neutrophil elastase (aNE) analysis used in the whole thesis. These data allowed us to reduce the potential heterogeneity in microbiome and inflammation results between different studies.

2) Then, we identified two groups of adults with bronchiectasis with different microbiome diversity levels. The low diversity group was found to be enriched in *Pseudomonas*, *Staphylococcus* and *Streptococcus*. Patientswere tested for univariate and multivariate analysis in order to understand clinical characteristics that may be predictors of low microbiome diversity. We identified FEV_1%_predict.<50, radiology and Primary ciliary dyskinesia (PCD) as risk factors for low microbiome diversity.

From these data, we decide to focus on aetiologies, and specifically on PCD and immunodeficiency. Considering PCD, data from a preliminary study matching these patients with idiopathic or post infective ones for sex, age and FEV_1%, did not identify a clear association between aetiology, microbiome and inflammation, however, a trend could be observed.

The second aetiological analysis was made on primary immunodeficiency, in comparison with idiopathic bronchiectasis. These two groups differed in terms of alpha diversity and of quantification of *H. influenza*, respectively lower and higher in the immunodeficiency one. Data on both PCD and immunodeficiency represent preliminary and promising results for the development of specific future multicentric studies for the characterization of sputum microbiome and inflammation in bronchiectasis aetiologies.

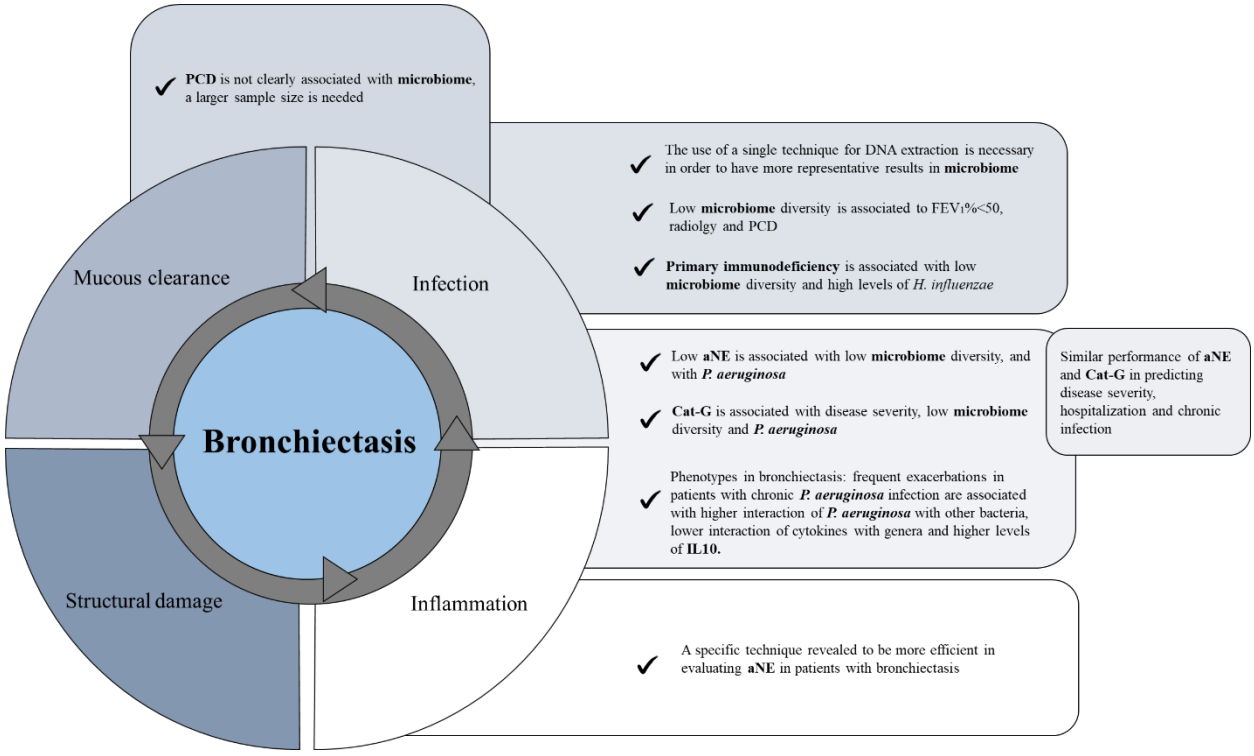
3) The following section of this thesis focused on neutrophilic infection, associating aNE in bronchiectasis with microbiome analysis. We found evidences of low microbiome diversity in patients with high levels of aNE. *Pseudomonas* in microbiome analysis and *P. aeruginosa* in

molecular biology (both detection and bacterial load) and chronic infection were associated to high levels of aNE.

Moreover, we considered Cat-G as a possible biomarker for bronchiectasis. Cat-G was found to be associated to disease severity, radiological severity, quality of life and chronic infection from the clinical point of view. Cat-G was also associated with low microbiome diversity and *P. aeruginosa* molecular detection. Finally, Cat-G and aNE resulted to be similar in predicting severe BSI, severe exacerbation and chronic infection in bronchiectasis patients in stable state.

4) We finally decided to focus on clinical phenotypes. Studies so far underlined the correlation between *P. aeruginosa* and neutrophilic inflammation. A heterogeneity in number of exacerbations was reported both from literature and clinical experience. Patients with chronic *P. aeuruginosa* with different frequency of exacerbations were enrolled for this preliminary study. No differences in terms of microbiome were found among the two study groups, while differences in terms of number of interactions of *Pseudomonas* with other genera and in the interaction between cytokines and microbial effectors were identified among the two groups at association network analysis.

Figure 1. Results outline



This thesis and the scientific papers arising by it represent a step towards the understanding of the relationship among microbiota, inflammation and patients' clinical characteristics in adults with bronchiectasis.

Since every chapter includes a specific discussion, this final discussion will cover an overall discussion underling unanswered questions and research opportunities arising from this experience.

6.2 The role of sputum dysbiosis in bronchiectasis

Microbiome is central in chronic respiratory disease. As discussed, decrease in pulmonary diversity seems to be related to disease *status* and disease severity in many pulmonary diseases. Moreover, our findings identified altered lung function and radiological severity as disturbers of airway diversity. Dysbiosis is also linked to inflammatory status in lungs, fitting in the model of the vicious vortex of bronchiectasis pathophysiology [1].

Pulmonary microbiome is very important in immune system training. Its development starts just after birth and it is in continuous renewal due to the direct exposure to the environment [2]. The airway microbiome, as the gut one, has been reported to have an effect in shaping lung immunity and there are evidences of dysbiosis affecting both innate and adaptive immunity [3].

Microbial dysbiosis may be identified as a treatable trait in bronchiectasis. Treating dysbiosis is an effective strategy in intestinal department. The use of probiotics is just one of the techniques used by clinicians and researchers to treat intestinal and non-intestinal diseases like irritable bowel syndrome, celiac disease, and inflammatory bowel disease, however it also include the treatment of obesity, metabolic disorder, cardiovascular syndrome, allergy, and asthma [4]. The strategies adopted by the scientific community goes from probiotics administration, the direct administration of symbiotic bacteria able to colonize the intestinal district and avoiding the installation of dangerous bacteria, to bacterial transplantation using bacterial consortia or direct faecal transplant, to phage therapy having a modulatory effect on the microbiome (still under study). Microbiome manipulation, mostly in early life should be a future target for the prevention of respiratory diseases, and, furthermore, targeting respiratory microbiome may be a future strategy for precision medicine focused on treatable traits in bronchiectasis.

Moreover, airway microbial community is not isolated in the human body. A cross-talk between lung and gut is known. Both sites are exposed to the outer environment and to immigration-emigration routes of bacteria. The presence of microbial flora in the two sites is known to prime host immune system [5]. The nature of this cross-talk is controversial; however, evidence based experiences reported the acquisition of information from microorganisms in gut shaping immune response in lung. The direct bacterial translocation of microorganisms between organs is still debated [6]. Moreover, evidences show that major inflammation in one of the two sites is able to transform microbiota in the other site [5].

Bacteria are not the only microorganisms colonizing our body. Both gut and lung mycobiota has been recently identified. Mycobiota and microbiota strictly interacts and cooperate as a community and may also cooperate in biofilm formation. Moreover, mycobiota seems also to adjuvate bacteria in shaping host immune system [7, 8]. The gut-lung interaction, as well as interaction among mycobiota and microbiota is poorly understood in bronchiectasis.

The study of the interplay of these two sites and the inclusion of other microorganisms' community, such as mycobiome may lead to the identification of new treatable traits in bronchiectasis.

6.3 The role of *P. aeruginosa* in bronchiectasis

The role of *P. aeruginosa* is more and more central in bronchiectasis, however,it opens to new questions concerning its relation to microbiome and host immune response.

The association of *Pseudomonas* and bacterial diversity is constant in all the hereby-presented experiences. *P. aeruginosa* is associated in bronchiectasis to increased disease severity and poor outcomes even if the only presence of *Pseudomonas* is not enough to fully predict patient's prognosis.

P. aeruginosa is biofilm forming bacterium, very resistant and difficult to treat, mostly diffused in our area (south Europe). *P. aeruginosa* is also able to directly inhibit the growth of other bacterial species like *H. influenzae*[9]. Patients with *P. aeruginosa* chronic infection may have very different exacerbation patterns [10]. Our experience identified the difference among the two groups of patients in the interactions between bacteria and in the interplay among microbiome and immune response.

The understanding of the interactions among bacteria in complex niches is a hot topic nowadays. In order to investigate the role of each genus in the pulmonary niche, specific"-omic" studies, including metatranscriptomic and metabolomic may be combined to microbiome analysis and, eventually, associated to trascriptomicsinvestigating host immune response to microbial colonization [11]. Microbiome analysis through 16s rRNA gene sequencing does not give per se information about specific interactions that may arise among bacteria, however it may open the way to the analysis of co-occurrence associations.We may speculate that *P. aeruginosa* is able to compete with other genera for the reduced resources in bronchiectasis lungs and is able to predominate on other bacteria through inhibitory strategies [12]. Rogers *et al.* reported a strong specific competition among *P. aeruginosa* and *H. influenzae*, able to lead to mutual exclusion

when one of the two species is prevalent in bronchiectasis airways [12]. Association analysis among bacteria in *P. aeruginosa* chronically infected patients, report a negative association of *Pseudomonas*with all the genera in the network, including *Haemophilus*in both frequent and non-frequent exacerbators.Furthermore, the largest number of negative associations with *Pseudomonas*was found between frequent exacerbators. The condition of frequent exacerbator seems to be associated to *Pseudomonas* ability in inhibiting the other bacterial genera. Bacterial dynamics in complex bacterial communities are largely studied.Quorum sensing is a bacterial strategy of communication and interaction among complex communities, which allows bacteria to coordinate group behaviour that may also give an ecological advantage in competition [13]. It drives many bacterial behaviours, including metabolic changes and antimicrobial compounds production [13]. Among others, *P. aeruginosa* producessmall antimicrobialsable to influence gene expression in many bacterial species [13]. These behaviours may be maypartially explain our results in bronchiectasis; however,the host immune system may have an active role in this mechanism.

The focus on this pathogen and on its interaction with both microbiome and host inflammation may provide important insights in bronchiectasis disease.

6.4 The role of neutrophilic inflammation in bronchiectasis

Neutrophilic inflammation was reported to be a predominant pattern in bronchiectasis airway. Biomarkers of local neutrophilic response as active neutrophil elastase and active cathepsin G are identified to be associated with both poor patients' condition and low microbiome diversity. The biomarkers analysed have comparable association with both clinical and microbiological component in bronchiectasis.

6.5 Aetiologies

Aetiological heterogeneity is one of the hot topics in bronchiectasis nowadays. As discussed in the general introduction, the definition of the aetiology is very important for the identification of treatable traits. The aetiology generally determines how patients enter in the vicious cycle of bronchiectasis pathophysiology, however few is known about the underlying pathophysiological mechanisms of the different conditions. Interestingly, PCD, a genetic disease that may lead to the development of bronchiectasis, emerged from these studies as risk factor for low microbiome diversity. Microbiome is poorly studied in the different aetiologies and only one descriptive study from 2013 is reported in literature on PCD microbiome [14]. PCD is a rare condition, and

the enrolment of these patients in research studies is difficult [15]. Moreover, the study of immunodeficiency seemed to be very promising considering the role of airways microbiome in shaping local immune system. The identification of differences between aetiologies may lead to the understanding of new important mechanisms underlying bronchiectasis condition.

6.6 Precision medicine and bronchiectasis

The identification of a new inflammatory phenotype that may be associated to peculiar microbiological situation and specific patients' characteristics may be very important in a precision medicine approach in bronchiectasis. Bronchiectasis is a very heterogeneous disease, associated with different clinical phenotypes, showing a plethora of symptoms and specific biological endotypes.

The specific characterization of underling biological characteristics in bronchiectasis may lead to a deeper understanding of this chronic respiratory disease from the biological point of view. Furthermore, a deep characterization of patients may lead to the identification of new targets for personalized treatment and to a higher awareness of bronchiectasis heterogeneity. It would give researchers new tools to better shape clinical trials including the most suitable patients also from the microbial community point of view and the identification of clinical characteristics associated to low microbiome diversity may identify a group of patients that could benefit from a microbiome-targeted therapy.

6.7 Strength of this thesis project

The aim of this thesis was to evaluate in the widest way possible lung microbiome and inflammation topic in bronchiectasis. 1) We touched several topics, from microbiome characterization based on neutrophilic inflammation, using one known and one putative biomarker for disease severity in bronchiectasis, to the identification of risk factors for low microbiome diversity. Moreover, we considered some promising aetiologies and we tried to understand in detail the difference among two very similar groups of patients (frequent and non-frequent exacerbators with chronic *P. aeruginosa*). 2) The bronchiectasis cohorts in this thesis are among the biggest published in bronchiectasis so far for microbiome analysis. 3) Each study included a translational and a clinical evaluation. 4) Some of the sections of this thesis were published in peer reviewed journals, others will be part of upcoming publications. 5) One of the studies included European centres in order to increase data generalizability. 6) Most of the studies were possible due to a strict interaction among study groups all over the world and by the specific skills acquired during both a visiting period in Dundee (UK) and a fellowship in New York (USA).

6.8 Limitations of this thesis project

The major limitations of this thesis are 1) The cross-sectional nature of all the studies hereby included. The inclusion of samples at different time point and microbiome modifications during exacerbation would have enriched this experience. 2) Moreover, we only studied microbiome, not considering viruses or fungi colonizing bronchiectasis airways. 3) We acknowledge that a larger evaluation of inflammatory biomarkers may be used, considering both neutrophilic and Th2 inflammatory response. 4) The use of sputum as a matrix of interest opened our microbiome evaluations to possible upper airways contamination. As we discussed above, the use of sputum kept our studies closer to clinical practice and allowed us to enrol up to 185 patients for study in our centre. The use of deep lung sampling methods would be more restrictive and less representative of the bronchiectasis population. 5) Most of the experiences included in this thesis were monocentric. The monocentric nature may limit the generalizability of these findings. Multicentric studies will be needed in order to confirm our findings. 6) The low numbers of patients treated with long term antibiotics enrolled in our studies did not allow us to produce data on the effect of these drugs on pulmonary microbial community. Moreover, the only long term macrolide used in our population is azithromycin, that would further limit this analysis. Larger studies are needed to investigate this association.

6.9 Conclusions

The hereby-presented translational studies draw the attention to microbiome and inflammation in bronchiectasis. 1) After a methodological approach to identify the technical steps needed for microbiome and aNE studies, we focused on microbiome and inflammation in sputum of adults with bronchiectasis. We faced these topics from many points of view. 2) The study of sputum microbiome in bronchiectasis lead to the identification of PCD, FEV_1% and radiology as associated to microbiome diversity. 3) Aetiologies were already one of our fields of interest, however, a preliminary study reported a trend in microbiome diversity among PCD and idiopathic or post-infective. An *ad hoc* multicentrical study is needed to fully assess the research question. The other interesting aetiology was immunodeficiency, being airways microbiome involved in local immune system training. Our study identified the association among low diversity and high *H. influenzae* load with primary immunodeficiency. 4) Furthermore, we characterized microbiome in patients with different levels of a known biomarker for bronchiectasis, aNE and Cat-G. In both these studies, we associated high levels of the neutrophilic biomarker with low diversity and *P. aeruginosa*. 5) Finally, an association network analysis was used to deeply investigate the differences in microbiome and inflammation in patients with chronic *P. aeruginosa* experiencing different exacerbations rates.

This thesis project is trying to increase our knowledge into bronchiectasis pathophysiology by describing sputum microbiome and neutrophilic inflammation. This characterization may bring to the identification of new biomarkers for patients' stratification and treatment needed from both the clinical and scientific community. Nevertheless, the data hereby shown are not enough to fully understand bronchiectasis disease and further studies will be needed to completely unravel these topics.

We hope that this thesis will give an insight in the complexity of this disease, opening the way to the identification of new treatable traits and that this may be a step towards the precision medicine approach in bronchiectasis.

6.10 References

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7 Scientific publication arising from this thesis

Peer-reviewed publications

Oriano M, Gramegna A, Terranova L, Sotgiu G, Sulaiman I, Ruggiero L, Saderi L, Wu B, Chalmers JD, Segal LN, Marchisio P, Blasi F, Aliberti S. Sputum Neutrophil Elastase associates with microbiota and *P. aeruginosa* in bronchiectasis. Eur. Respir. J. [Internet] European Respiratory Society; 2020; Available from: https://erj.ersjournals.com/content/early/2020/05/22/13993003.00769-2020.

Oriano M, Terranova L, Sotgiu G, Saderi L, Bellofiore A, Retucci M, Marotta C, Gramegna A, Miglietta D, Carnini C, Marchisio P, Chalmers JD, Aliberti S, Blasi F. Evaluation of active neutrophil elastase in sputum of bronchiectasis and cystic fibrosis patients: A comparison among different techniques. Pulm. Pharmacol. Ther. 2019; 59: 1–6.

Oriano M, Terranova L, Teri A, Sottotetti S, Ruggiero L, Tafuro C, Marchisio P, Gramegna A, Amati F, Nava F, Franceschi E, Cariani L, Blasi F, Aliberti S. Comparison of different conditions for DNA extraction in sputum - a pilot study. Multidiscip. Respir. Med. Multidisciplinary Respiratory Medicine; 2019; : 1–8.

Abstracts at international meetings

Participation to the ERS International Congress 2019, Madrid with:

Comparison of different techniques to evaluate active neutrophil elastase in sputum of bronchiectasis and cystic fibrosis patients Martina Oriano, Leonardo Terranova, Giovanni Sotgiu , Laura Saderi , Angela Bellofiore, Mariangela Retucci, Cinzia Marotta, Andrea Gramegna , Daniela Miglietta, Chiara Carnini , Paola Marchisio , James D. Chalmers, Stefano Aliberti , Francesco Blasi

Participation to the ERS International Congress 15-19 September 2018, Paris with:

What is the best technique to extract bacterial DNA from sputum? Martina Oriano, Leonardo Terranova, Luca Ruggiero, Camilla Tafuro, Antonio Teri, Lisa Cariani, Elisa Franceschi, Francesco Amati, Andrea Gramegna, Martina Contarini, James D Chalmers, Paola Marchisio, Stefano Aliberti, Francesco Blasi (Poster discussion)

8 Addendum

During the PhD training, the author had the privilege to be involved in, or contribute to, different other projects in the field of bronchiectasis research. This work was outside the scope of the main project and is therefore mentioned in addendum.

Peer-reviewed publications

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- An extensive bundle of tests is needed to detect treatable causes of bronchiectasis (Bx) Elisa Franceschi, Stefano Aliberti, Manuela Seia, Luigi Porcaro, Vera Bianchi, Enza Consalvo, Angelo Corsico, Ilaria Ferrarotti, Michele Gaffuri, Luca Roncoroni, Barbara Vigone, Andrea Gramegna, Martina Contarini, Francesco Amati, Noemi Borsa, Fabrizio Nava, Nicolò Vanoni, Angela Bellofiore, Cesare Del Monaco, Leonardo Terranova, Martina Oriano, Lisa Cariani, Flavio Caprioli, Mario Nosotti, Francesco Blasi (Poster)
- Antimicrobial peptides and airway bacterial colonization in bronchiectasis Oriol Sibila, Lídia Perea, Elisabet Cantó, Guillermo Suarez-Cuartin, Ana Rodrigo-Troyano, Leonardo Terranova, Martina Oriano, Jordi Giner, Silvia Vidal, Stefano Aliberti, James D Chalmers (Poster)

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9 Italian Abstract

Le bronchiectasie sono una malattia respiratoria cronica caratterizzate da una dilatazione irreversibile dei bronchi in un contesto di sindrome cronica per la maggior parte caratterizzato da tosse e produzione di espettorato giornalieri e frequenti riacutizzazioni polmonari. La patofisiologia delle bronchiectasie è associata al ciclo vizioso di infezione, infiammazione, clearance del muco e danno polmonare. La comunità microbica e l'infiammazione hanno un ruolo centrale nelle malattie respiratorie, tuttavia pochi sono i dati riportati nelle bronchiectasie. L'obiettivo di questo progetto di dottorato è stato lo studio del microbioma respiratorio e dell'interazione del microbioma con la risposta infiammatoria dell'ospite in pazienti adulti con bronchiectasie in fase di stabilità. Alcuni studi sono stati condotti al fine di rispondere a questa domanda di ricerca.

Per prima cosa abbiamo riportato la selezione della metodologia per l'analisi di microbioma e della elastasi neutrofila attiva (aNE) nell'espettorato.

In seguito, sono stati individuati due gruppi con diversi livelli di diversità al microbioma. Il gruppo con più bassa diversità è stato riportato come arricchito in *Pseudomonas*, *Staphycococcus* e *Hemophilus*. L'analisi multivariata ha individuato $FEV_1\%<50$, radiologia e discinesia ciliare primitive (PCD) come indipendentemente associate a bassa diversità di microbioma. La sezione seguente rappresenta uno studio preliminare focalizzato su alcune eziologie di bronchiectasie, specificamente PCD e immunodeficit. Lo studio sulla PCD non ha identificato una chiara associazione tra microbioma infiammazione e eziologia, individuando tuttavia un trend nei dati. In seguito, è stato considerato l'immunodeficit in comparazione con bronchiectasie idiopatiche. L'immunodeficienza primaria è stata associata a una minor alfa diversità di microbioma e ad una maggior presenza di *H. influenzae*.

Il capitolo quattro si è focalizzato sull'infezione neutrofilica, associando l'aNE all'analisi del microbioma. Sono state così individuate evidenze di una più bassa diversità e presenza di *P. aeruginosa* in pazienti con alti livelli di aNE.

Lo studio seguente si è focalizzato sulla Catepsina G attiva (Cat-G), un possibile nuovo biomarcatore nelle bronchiectasie. La Cat-G è stata associata a gravità di malattia, gravità radiologica, qualità della vita e infezione cronica. È stata inoltre individuata una associazione con bassa diversità di microbioma e rilevamento di *P.aeruginosa*.

Infine, abbiamo analizzato in microbioma e l'infiammazione locale in pazienti adulti con bronchiectasie con diagnosticata infezione cronica da *P. aeruginosa*. L'analisi del network di associazione ha identificato differenze in termini di associazione di *Pseudomonas* con altri generi e di citochine infiammatorie e batteri tra frequenti e non frequenti riacutizzatori.

I risultati di questa tesi hanno bisogno di ulteriori studi e conferme. Speriamo che questa tesi possa rappresentare un passo in avanti verso una miglior comprensione del ruolo di microbioma ed infiammazione nelle bronchiectasie che può portare a una miglior caratterizzazione degli endotipi in questa malattia, e individuazione di nuovi target terapeutici risultando in avanzamenti nell'approccio di medicina personalizzata per i pazienti con bronchiectasie.

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