# The airway microbiota of stable COPD

Association with exacerbation frequency and the risks associated with bronchoscopic data collection

Elise Orvedal Leiten

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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UNIVERSITY OF BERGEN

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# Table of contents

# Terms and abbreviations

| | |
|---|---|
| 16S rRNA | RNA component of the 30S subunit of a prokaryotic ribosome |
| AECOPD | Acute Exacerbations of COPD |
| Adonis | Implementation of PERMANOVA in R |
| AERIS study | Acute Exacerbation and Respiratory InfectionS in COPD study |
| ALDEx2 | ANOVA-Like Differential Expression Analysis, a DA-test |
| Alpha diversity | The level of diversity found within a single sample |
| ANCOM | Analysis of composition of microbes, a DA-test |
| ANCOM-BC | ANCOM with bias correction |
| ASV | Amplicon sequence variant |
| BAL | Bronchoalveolar lavage |
| BAL2 | Second fraction of BAL |
| Beta diversity | The level of diversity or dissimilarity found between samples. Used to examine whether samples within a group are more similar to each other than those in another group. |
| BMI | Body Mass Index |
| BTS | British Thoracic Society |
| CAT | COPD Assessment Test |
| Clavien-Dindo | A complication assessment tool |
| CONSORT | Consolidated Standards of Reporting Trials |
| COPD | Chronic Obstructive Pulmonary Disease |
| COPDMAP study | Chronic Obstructive Pulmonary Disease Medical Research Council/Association of the British Pharmaceutical Industry study |
| CSS | Cumulative sum scaling |
| CTCAE | Common Terminology Criteria for Adverse Events, a complication assessment tool |

| | |
|---|---|
| DA-tests | Differential abundance tests, statistical tests developed for identification of features that significantly differ in abundance (or is otherwise differentially expressed) between groups of interest |
| DADA2 | Divisive Amplicon Denoising Algorithm version 2, a denoising algorithm |
| DDA | Differential Distribution Analysis, a DA-test |
| Deblur | A denoising algorithm |
| Decontam | A bioinformatic tool for contaminant removal |
| Diversity | The richness and/or distribution of taxa in a sample and similarity/dissimilarity of taxonomic composition between samples |
| EBB | Endobronchial biopsies |
| ECG | Electrocardiogram |
| FastTree2 | A tool that infers approximately-maximum-likelihood phylogenetic trees from alignments of nucleotide or protein sequences |
| FB | Flexible bronchoscopy |
| FDR | False discovery rate (Benjamini-Hochberg method) |
| $FEV_1$ | Forced Expiratory Volume in 1 second |
| FVC | Forced Vital Capacity |
| Gneiss | Balance test, a DA-test |
| GOLD | Global Initiative for Chronic Obstructive Lung Disease |
| HOMD | Human Oral Microbiome Database |
| HUH | Haukeland University Hospital |
| ICS | Inhaled corticosteroids |
| ITS1 | internal transcribed spacer 1 |
| LABA | Long-acting $beta_2$ agonist |
| LAMA | Long-acting muscarine antagonist |
| LEfSe | Linear discriminant analysis effect size |
| LLN | Lower limit of normal |

| | |
|---|---|
| Mafft | Multiple Alignment using Fast Fourier Transform, is a high speed multiple sequence alignment program |
| MeSH | Medical Subject Heading |
| MicrobiomeDDA | R package, see DDA |
| MicroCOPD | Bergen COPD Microbiome Study |
| MicroDecon | A bioinformatic tool for contaminant removal |
| MicroILD | The Microbiome in Interstitial Lung Disease study |
| mMRC | Modified Medical Research Council scale |
| MSRP | Medical Student Research Programme (Forskerlinjen) |
| NCS | Negative control sample |
| NCBI BLAST | National Center for Biotechnology Information Basic Local Alignment Search Tool |
| OTU | Operational Taxonomic Unit |
| OW | Oral wash |
| $PaCO_2$ | Partial pressure of carbon dioxide |
| $PaO_2$ | Partial pressure of oxygen |
| pBAL | Protected BAL |
| PBS | Phosphate-buffered saline |
| PCoA | Principal Coordinates Analysis |
| PCR | Polymerase chain reaction |
| PERMANOVA | Permutational multivariate analysis of variance |
| PICO | Population - Intervention - Comparison - Outcome |
| PRISMA | Preferred Reporting Items for Systematic reviews and Meta-Analyses |
| PROSPERO | International Prospective Register of Systematic Reviews |
| q2 | QIIME2, abbreviation in plugins |
| QIIME2 | Quantitative Insights Into Microbial Ecology 2 |
| qPCR | Quantitative PCR |
| Rarefaction | A process which subsamples each sample to a given sequencing depth without replacement. Samples with a sequence count below the given value is discarded. |

| | |
|---|---|
| RCT | Randomised Controlled Trial |
| Relative abundance | The proportion of that feature in relation to the sum of features in that sample |
| rPSB | Protected Sterile Brushes from the right lung |
| SAFTEE | Systematic Assessment for Treatment of Emergent Events, a complication assessment tool |
| SD | Standard deviation |
| SPIROMICS | Study of COPD Subgroups and Biomarkers |
| $SPO_2$ | Peripheral capillary oxygen saturation |
| spp | Species, plural |
| SVC | Superior vena cava syndrome |
| SVL | Small volume lavage |
| Taxon | A taxonomic group of any rank, such as a species, family, or class. |
| TBB, TBLB | Transbronchial biopsies, transbronchial lung biopsies |
| Trimmomatic | A read trimming tool for Illumina NGS data |
| V3-V4 | Variable regions 3 and 4 of the 16S rRNA gene |
| VSEARCH | Vectorized search, a bioinformatic tool |
| Zero-inflation, sparsity | Refers to the very large number of zeros in the feature table, which could be caused by both under-sampling and true biological differences |

# Scientific environment

This thesis is anchored in the Bergen COPD Microbiome study (MicroCOPD). The study was conducted at the Department of Thoracic Medicine at Haukeland University Hospital, by the Bergen Respiratory Research Group. The group is led by professor Tomas Mikal Lind Eagan.

I have been affiliated with the Department of Clinical Science, Faculty of Medicine, University of Bergen. The work with this thesis started as a project within the Medical Student Research Programme (MSRP, Norwegian: Forskerlinjen). MSRP includes one year of full-time research and later part-time research in addition to the ordinary medical degree programme. I became a PhD candidate in 2018. The PhD work, including the MSRP, was funded by the University of Bergen.

During MSRP, my main supervisor was Rune Nielsen, associate professor, MD at the Department of Clinical Science, Faculty of Medicine, University of Bergen.

As a PhD candidate, my main supervisor has been Tomas Mikal Lind Eagan, professor, MD at the Department of Clinical Science, Faculty of Medicine, University of Bergen.

Co-supervisors have been associate professor Rune Nielsen and professor Harald Gotten Wiker, both at the Department of Clinical Science, Faculty of Medicine, University of Bergen.

# Acknowledgements

In my research group, this section often depicts the story of how the PhD candidate was tricked into doing research. Typically, the person responsible for deceiving these young and aspiring medical doctors is professor Per Bakke. In my case, the story goes a bit differently. As the MicroCOPD study was being planned, Per decided that the project should involve a student in the Medical Student Research Programme (MSRP). Other involved researchers were, understandably, rather sceptical towards incompetent second-year medical students. Per insisted. And for that, Per, I am grateful! Anyways, so it happened, that post doc Rune Nielsen went, perhaps somewhat unwillingly, to the MSRP information meeting to recruit a student…

Rune, you can be very convincing. Your persuasive powers are the reason why the MicroCOPD study ended up having not just one, but *two* useless medical students on board. As supervisor for Einar Marius and me throughout the MSRP and medical school you've been an inspiration. You are kind, smart, funny and ambitious (also on behalf of others). Some might say you talk too much, but I think you are excused. It does require a great deal of talk if one wants to delve into research related topics in addition to solving world peace. Thank you for all your help and guidance!

Dear Tomas. I feel very lucky to have you as my supervisor. You have an astonishing work capacity, and you are extremely structured, thorough and knowledgeable. This is in part what makes you such a great researcher and teacher. I have also admired and appreciated how you always maintain a calm and reassuring attitude, even when faced with a rather hysterical PhD student. Thank you for interesting discussions, valuable comments, and prompt email replies – all delivered with a ton of patience!

Harald, my third supervisor and go-to microbiologist. Thank you for introducing me to laboratory work, for your contribution to paper III and for your feedback on this thesis.

Einar Marius. One of the purposes of a PhD is to make the student an independent researcher. Doing more or less everything together, I guess we've been as independent as the parties of an old married couple. This whole research process has taught me a lot of different things and given me some useful skills and many precious experiences. But, if I had to choose one favourite result of my research, it is the friend that I got in you! Thank you for everything!

Thanks to the Thoracic Department for facilitating clinical research. The Bergen Respiratory Research Group has provided a safe and including working environment, and I would like to thank everyone in it! Eli deserves a special thanks. I've always been welcome in your office, and I guess I have taken advantage of that. Thank you for having the answers to everything! Ane, thank you for interesting discussions and for social meetings, even in maternity leave! Thanks also to Anders, Andreas, Bahareh, Bente, Bernt, Birger, Christina, Christina, Christine, Einar, Fabian, Gunnar, Hilda, Hildegunn, Ingvild, Jon, Kristel, Kristina, Lise, Lucia, Margrethe, Marianne, Marit, Marta, Maryia, Rajesh, Randi, Randi, Solveig, Stine, Sverre, Tharmini, Themina, Tiina, Tove, Tuyen, Øistein and Øystein. You have contributed to collection and processing of data in the MicroCOPD study, provided feedback on manuscripts and presentations, taken part in fruitful discussions and social meetings, or all of the above.

There are a lot of people to thank also outside the research group. Obviously, the participants in the MicroCOPD study deserves a big thank you! Thanks also to the University of Bergen, and especially thanks to the MSRP! The opportunity to start my research early has really been an advantage! It has also been of great value to be part of this environment of students. You didn't always make the choice of doing research alongside medical school seem any more reasonable, but at least I've felt reassured knowing that we were all in the same boat. A special thanks to Jian Hao. We've been friends ever since we started med school, and in a way, I feel like we have "grown-up" together. You are good at everything you decide upon doing, so logically I was very pleased when you decided to start dancing. I hope you'll dance with me at my defence

party!

The Institute of Clinical Science has a policy of randomly allocating their employees all across campus. I think it serves a public health purpose; it forces everyone to a minimum exercise when climbing stairs or walking the long underground corridors of the hospital in order to meet their colleagues. As a PhD candidate I've been seated at the Centre for Nutrition(!), and I would like to thank everyone there. You make up a great workplace! A special thanks to my office mate Ester. When I am stressed out, your comforting words always lower my blood pressure.

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My dear little Arnfinn. By coming into this world, you delayed my work a little bit. Thank you for postponing the submission and defence of this thesis until the covid related restrictions were lifted. Also: You are my favourite person. You turned my life up-side-down and are the best thing that ever happened to me.

# Abstract

Background

Acute exacerbations of COPD are an important cause of mortality and morbidity in patients with COPD. It is incompletely understood why some COPD patients experience frequent exacerbations, while others rarely or never exacerbate. Studies have suggested that the microbiome of the lungs is different in patients with different exacerbation frequencies. Most studies use sputum samples prone to contamination from the upper airway. Bronchoscopic sampling could improve the quality of the samples, but is a more invasive approach.

Aims

The overall aim of the PhD project is to investigate if the airway microbiota in subjects with stable COPD is associated with exacerbation frequency and to assess the complications and discomforts (including rates and predictors) associated with bronchoscopic data collection in participants with and without COPD.

Materials and Methods

For the first paper, we performed a systematic literature search on complications and discomfort of non-therapeutic bronchoscopy in PubMed. Titles and abstracts of retrieved search hits were sorted according to inclusion and exclusion criteria.
The second and third paper uses data collected in the Bergen COPD Microbiome Study (MicroCOPD). Individuals with and without COPD underwent bronchoscopy including protected bronchoalveolar lavage (BAL) (in participants with $FEV_1$ >30% of predicted), protected specimen brushes (PSB), small volume lavage, and in 1/3 of bronchoscopies, endobronchial biopsies. In addition to bronchoscopic sampling, participants provided oral wash samples. For each bronchoscopic procedure, there was one negative control sample of the phosphate-buffered-saline used for the microbial samples. Some participants underwent more than one bronchoscopy. Light sedation with alfentanil was offered to participants. Immediate complications, defined as any event requiring an unplanned intervention or early termination of the procedure, were

recorded. Participants were interviewed after a week regarding discomfort, respiratory symptoms and fever sensation. Participants with COPD were followed with telephone interviews every three months for one year regarding exacerbations. Microbial samples and negative controls went through laboratory processing including DNA extraction, PCR and sequencing of the 16S rRNA gene. Extensive bioinformatic processing of sequencing data and microbiota analyses were performed using QIIME2 and R. Pre-processing included bioinformatic identification and removal of contaminant sequences. We them compared bacterial taxonomy and alpha and beta diversity in individuals with and without COPD exacerbations in the follow-up.

Results

Bronchoscopy is generally a safe procedure with low mortality and few severe complications, but the literature shows a wide range of specific complication rates, and it was not possible to conclude on discomfort or predictors.

In MicroCOPD, 239 participants underwent bronchoscopy once, 61 underwent more than one bronchoscopy. Complications occurred in 25.9% of first bronchoscopies. The rate of potentially severe complications was 1.3%. Participants with COPD experienced more dyspnoea than participants without lung disease. Sedation and lower age were associated with less complications. 47.7% reported fever. Discomfort was associated with fever, dread of bronchoscopy, high COPD Assessment Test score, and never-smoking. Complications and fever in a first bronchoscopy were often predictive for complications and fever in a second bronchoscopy. We found no difference in alpha and beta diversity between participants with and without COPD exacerbations, and no ASV or genus was found to be consistently differentially abundant or distributed between the groups.

Conclusions

Bronchoscopy is a generally safe procedure, even in research into COPD, but is not free of risk. Bronchoscopy was associated with frequent need for unplanned interventions, discomfort and fever sensation in MicroCOPD. We found no association between the lung microbiota at stable state and exacerbations of COPD.

# List of Publications

Paper I

Leiten EO, Martinsen EM, Bakke PS, Eagan TM, Grønseth R.
Complications and discomfort of bronchoscopy: a systematic review.
Eur Clin Respir J. 2016 Nov 11;3:33324.

Paper II

Leiten EO, Eagan TML, Martinsen EMH, Nordeide E, Husebø GR, Knudsen KS, Lehmann S, Svanes Ø, Bakke PS, Nielsen R.
Complications and discomfort after research bronchoscopy in the MicroCOPD study.
BMJ Open Respir Res. 2020 Mar;7(1):e000449.

Paper III

Leiten EO, Nielsen R, Wiker HG, Bakke PS, Martinsen EMH, Drengenes C, Tangedal S, Husebø GR, Eagan TML.
The airway microbiota and exacerbations of COPD.
ERJ Open Res. 2020 Aug 31;6(3):00168-2020.

# Introduction

The thesis is part of the Bergen COPD Microbiome Study (MicroCOPD), and is based on three papers covering, broadly, two main topics: 1) safety of bronchoscopy, the sampling methodology chosen in MicroCOPD, and 2) a potential association between airway microbiota and exacerbations of chronic obstructive pulmonary disease (COPD).
In this chapter, I provide some background information intended to help the reader understand the objectives, methods, results and discussion of the thesis.

## Chronic Obstructive Pulmonary Disease

According to the Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD), COPD should be diagnosed based on findings of both *obstructed airflow* and persistent respiratory *symptoms* (1). In this chapter, I present some known risk factors, the pathophysiology, symptoms, diagnostic criteria for airflow obstruction, treatment, disease grading and phenotype classification.

### Risk factors

Tobacco smoking is the most important and preventable risk factor; however, indoor or occupational air pollution are also contributors to COPD (2). The risk of developing COPD increases with advancing age (3). In addition, individuals have different susceptibility towards developing the disease. Such a susceptibility can be caused by for instance genetic risk factors (especially alpha-1 antitrypsin deficiency) (4), asthma (5) and airway hyper-responsiveness (6), and disturbances in early lung growth and development (7).

**Pathophysiology**

The airflow limitation and symptoms of COPD are attributable to abnormalities in the bronchi, bronchioles and alveoli usually caused by exposure to noxious particles or gases. Exposure to such irritants leads to an inflammatory response, which is normal. In patients who develop COPD, this response is modified and the response turns into a chronic inflammation of the lung. Many factors might impact this inflammatory response and the course of COPD disease progression: the amount of oxidative stress (from inhaled noxious gases), the levels of proteases and anti-proteases, inflammatory cells, inflammatory mediators (growth factors, cytokines), exposure to infectious agents, as well as treatment drugs (8). The inflammatory response present in COPD is often characterised by infiltration and activation of neutrophils, macrophages and lymphocytes (9), somewhat similar to the response associated with bacterial pneumonia (10). Some patients have a more eosinophilic pattern (9). Inflammation followed by fibrosis development and mucus excretion directly leads to narrowing and destruction of the airways which gives limited airflow. The inflammation also induces damage to the parenchymal tissue, breaking down alveolar walls, resulting in the condition known as emphysema. Emphysema contributes to airflow limitation, and reduces gas exchange leading to hypoxaemia and hypercapnia. COPD is associated with hyperinflated lungs, a result of air being trapped from exiting on expiration. The pathological processes described above cause dyspnoea in COPD patients. In addition, hypersecretion of mucus leads to productive cough (1), which is the ground for diagnosing chronic bronchitis.

Importantly, COPD does not only affect the lung, but is indeed a systemic disease. Changes in respiration and ventilation affect the heart and circulatory system. The inflammation also has potential systemic effects. COPD patients frequently suffer from a wide range of comorbidities including cardiac disease, hypertension, anaemia, musculoskeletal dysfunction, diabetes, osteoporosis, cancer and psychiatric illness (11, 12).

## Symptoms

Although many population-based studies rely on obstructed airflow alone to define COPD, a diagnosis of COPD requires the presence of persistent respiratory symptoms. Typical symptoms are dyspnoea, cough and the production of sputum. Symptoms are often progressive. Many patients with COPD experience exacerbations, episodes of worsened respiratory symptoms (see Acute exacerbations of COPD) (1).

## Obstructiveness

Obstructive airflow in the airways is diagnosed and quantified by spirometry. Forced expiratory volume after 1 second ($FEV_1$) and forced vital capacity (FVC) is measured. The ratio between these measurements, $FEV_1$/FVC, can be used to determine if the respiration is obstructed. The cut-off separating obstructed airflow from normal airflow is set to a ratio of 0.70 (1). Of note, this fixed ratio criterion is debated, as it results in a considerable prevalence of obstruction in healthy individuals (especially elderly), as well as also being less sensitive in detecting early signs of airway disease in others (13, 14). Therefore, it has been suggested to use reference values from a general population to estimate the cut-off for an individual. Using the lower limit of normal (LLN) would mean that the 5% lowest values are defined as abnormal (14).

## Treatment

There is no cure against COPD. However, many measures can be taken to prevent the disease from progressing and to relieve symptoms (1). For treatment of COPD exacerbations, see “Acute exacerbations of COPD”. For maintenance therapy, both non-pharmacological and pharmacological treatment should be considered. All COPD patients who smoke should be advised and helped to quit, and other ongoing harmful exposures should be identified and eliminated. Specific recommendations for pharmacological interventions depend on disease severity. Most patients with COPD receive inhalation bronchodilators, usually a long-acting muscarinic antagonist

(LAMA) or a long-acting beta$_2$ agonists (LABA), or combination therapy. Inhaled corticosteroid (ICS) treatment is indicated in those with many exacerbations, high eosinophilic blood count and asthma. In addition to inhalation therapy, COPD treatment can include nutritional support, exercise, pulmonary rehabilitation, supplemental oxygen, non-invasive positive pressure ventilation, pneumococcal and influenza vaccinations, phosphodiesterase-4 inhibitors, oral glucocorticoids, mucolytics, theophylline and continuous antibiotic treatment (macrolides) (1).

## Grading COPD

Severity of obstruction in COPD is graded into four categories based on spirometry ($FEV_1$, in % of expected value). The grades are (from least to most severe airway obstruction) GOLD 1 ($FEV_1 \geq 80\%$), GOLD 2 ($FEV_1$ 50-79%), GOLD 3 ($FEV_1$ 30-49%) and GOLD 4 ($FEV_1 <30\%$). Since airway obstruction level alone does not necessarily reflect the disease severity, COPD patients can also be grouped according to a combination of symptom scores and frequency of moderate to severe exacerbations in the preceding year (1). The symptom scores being used are COPD Assessment Test (CAT) (15) and the modified Medical Research Council scale (mMRC) (16). There are four groups: A, B, C and D in which COPD patients are assigned to (1), like shown in Table 1.

**Table 1: Groups A-D for assessment of COPD symptoms and exacerbation risk.**

| **Exacerbations** | | |
|---|---|---|
| ≥ 2 moderate, or ≥ 1 severe | C | D |
| 0-1 moderate (no hospitalisation) | A | B |
| **Symptom score** | mMRC 0-1<br>CAT < 10 | mMRC ≥ 2<br>CAT ≥ 10 |

GOLD grade 1-4 and group A-D are used in combination. For instance, a COPD patient with a $FEV_1$ of 60 %, one exacerbation that did not require hospitalisation and high symptom scores will be classified as GOLD 2, group B.

**Phenotypes**

Unlike COPD GOLD grade and group, *COPD phenotype* is not a standardised term. In the literature, COPD phenotypes may refer to many different alternative categorisations of COPD patients. Examples of characteristics that are used to define COPD phenotypes include inflammation type (eosinophilia/neutrophilia in blood or sputum) (17, 18), response to bronchodilator treatment (19), predominance of emphysema or chronic bronchitis (19, 20), body composition (21), sex (22), comorbidities (23, 24), rapid lung function decline (25, 26) and whether or not the patients experience (frequent) exacerbations (27, 28). In this thesis, the frequent vs infrequent exacerbation phenotype is investigated. Often, the cut-off between frequent and infrequent exacerbator is set at 2 in the preceding year (29-35), but the cut-off can also be set at 1 (36, 37) or 3 (38).

## Acute Exacerbations of COPD

Persons with COPD may experience periods of worsened respiratory symptoms that necessitates additional therapy. These events are usually called acute exacerbations of COPD (AECOPD); often (and in this thesis) referred to simply as *exacerbations*. Exacerbations contribute substantially to reduced health related quality of life (39) and mortality (40) in COPD patients and are important drivers of disease progression (41). Exacerbations lead to increased health care usage and are the main driver of economic cost in COPD (42). Exacerbations vary in duration (typically days-weeks) (43). The respiratory symptoms typically seen in exacerbations include increased dyspnoea, increased volume and purulence of sputum, increased cough and wheezing (44). In severe cases, exacerbations can lead to respiratory failure (45).

The most important risk factor for developing an exacerbation is having experienced a previous exacerbation (27, 46-48), supporting the idea of an exacerbator phenotype, as described above. It is not entirely known why some patients with COPD are more susceptible to exacerbations. The exacerbation state is associated with increased inflammation (49). Exacerbations are considered to be caused or triggered mainly by respiratory viral infections, such as the common cold (rhinovirus) (50). Often, signs of bacterial infection or bacterial overgrowth is present. Bacterial infections could as well potentially trigger exacerbations, or represent secondary infection of an established exacerbation. Bacteria known to be associated with COPD exacerbations include *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* (51).

An exacerbation can be categorised as mild, moderate or severe; mild if it can be handled with patient-managed symptom treatment alone (increased usage of a short-acting bronchodilator), moderate if it requires additional treatment with antibiotics or systemic steroids and severe, if it leads to hospitalisation (1).

Systemic glucocorticoids are given to reduce the duration of an exacerbation and improve lung function. GOLD recommends 40 mg oral prednisolone a day for 5 days (1). Clinical experience dictates a sometimes longer course. Antibiotic treatment for 5-7 days is recommended with the presentation of both purulent sputum and either increased dyspnoea or increased sputum volume. It should also be considered for severe exacerbations requiring mechanical ventilation (1).

In summary, COPD exacerbations are inflammatory and often infectious states that for unknown reasons more often affect one part of the COPD population. Moderate and severe exacerbations are treated with antibiotic and anti-inflammatory drugs that have systemic consequences and significant side-effects. In addition, frequent antimicrobial treatment in the COPD population bears the potential of antimicrobial resistance. An improved understanding of what causes susceptibility towards COPD exacerbations and the frequent exacerbator phenotype could optimise the prevention and treatment of COPD exacerbations and potentially reduce antibiotic usage. It has been suggested

that airway bacteria present during stable phase COPD may play a role in the development of COPD exacerbations.

## Microbiome research

Due to recent advances in medicine, biology and bioinformatics, we have an understanding of the interaction between microorganisms and humans that is quite different from the one we had just a few decades ago. Microbes, including bacteria, viruses and fungi, are not viewed merely as commensals or pathogens of the human body anymore. Microorganisms live in symbiosis with their host and with each other. It is assumed that these communities within us impact our health. This even applies to diseases and conditions not traditionally considered to be of infectious origin, such as psoriasis (52), inflammatory bowel disease (53), irritable bowel syndrome (54), type 2 diabetes (55) and multiple sclerosis (56). Instead of focusing on single pathogens as causes of infectious disease, researchers now examine "healthy" microbial patterns in health, and discover disturbances in the community composition of microbes (a dysbiosis) in disease. Best studied are bacterial communities of the gut. Distinguishing between normal and disease-associated bacterial *compositions* could be a step in the direction of establishing new, improved and targeted treatment for a series of conditions.

### Defining the key terms

The vocabulary used in microbial community research has grown alongside with the rapid evolution of the field, resulting in confusing use (and misuse) of some common terms, including those used in this thesis. An editorial in the journal *Microbiome* attempted to provide clear definitions:

The authors defined the *microbiome* as “the entire habitat, including the microorganisms (bacteria, archaea, lower and higher eukaryotes, and viruses), their genomes (i.e., genes), and the surrounding environmental conditions.”

The *microbiota* was defined as “the assemblage of microorganisms present in a defined environment.” (57)

**Sequencing technology**

A key driver of development in this field has been the advancement of, and subsequent cost reduction of, the sequencing technology. Sequencing enables reading of genetic material (usually DNA) and has to a great extent replaced culture-dependent techniques. There are several different sequencing platforms (providers of sequencing technology), for instance Illumina, 454 and Ion Torrent. The different technologies vary when it comes to price and quality (different read-lengths, accuracy and throughput) (58).

The most common sequencing method in microbiota research is *marker gene amplicon sequencing* (also called marker gene sequencing or amplicon sequencing), in which only a specific target gene is PCR amplified and sequenced. For this to work, the marker gene needs to be present in *all* the organisms that we want to identify. The gene must be similar enough across all organisms that it can be identified as this particular gene in order for all organisms to be detected (conserved regions). At the same time, the marker gene has to include variable regions with alterations that allow for separation and classification of the different organisms. In bacteria, the marker gene for 16S rRNA is used. All bacteria share this gene that codes for the small-subunit ribosomal RNA locus, and the genetic information in the nine variable regions is different in different types of bacteria (59), although it cannot be used to classify bacteria beyond the genus level with certainty (60). Usually, only a part of the gene (including one-two variable regions) is amplified and sequenced (61).

An alternative to marker gene sequencing is shotgun sequencing, in which all DNA from all organisms in a sample is sequenced. Shotgun sequencing provides high resolution (taxonomic classification at species and strain level) and in addition functional profiling. Although there are continued cost reductions also in this technology, shotgun sequencing is still often prohibitively expensive, and the bioinformatic management and further analysis and interpretation of shotgun sequencing data is more challenging (62).

Although many of the principles explained in the following sections are valid for all types of sequencing data, it should be noted that this thesis is based on data and literature from studies using 16S rRNA gene sequencing, and that the introduction to data management in "Bioinformatic pre-processing" and "Microbiota analysis" reflects that.

**Bioinformatic pre-processing**

No sequencing machine provides clean, straight-forward sequencing data suited for direct analysis of taxonomy. The output requires bioinformatic handling for technical reasons and for quality control. For instance, the sequencing data has to be organised into separate features for later identification of different organisms. Some optional and required processing steps are listed in Table 2.

**Table 2: Examples of bioinformatic processing steps in the management of microbial sequencing data.**

| Processing step | Short description |
|---|---|
| Demultiplexing | Removal of barcodes/indexes used during sequencing and splitting of sequencing information into separate files for each sample. Is often performed by the sequencing facility. |
| Sequence quality control and feature table construction | Removes sequencing "noise", e.g., chimeras (artifact sequences formed by incorrect union of two or more biological sequences). Divides similar/identical sequences into feature entities, for instance amplicon sequence variants or operational taxonomic units and formats data into a table for further analysis. Different algorithms/software can be applied for this purpose, for instance DADA2(63) or Deblur(64). |
| Filtering | Feature tables can be filtered:<br>- As part of quality control: Removing features that only appear in few samples or that have few sequences, or features identified as contaminant sequences<br>- Down to only features or only samples of specific interest |
| Construction of a phylogenetic tree | Using the genetic information in the sequences, a tree relating the features to one another can be constructed and gives information on genetic similarity between features. |
| Assignment of taxonomy | Taxonomic identification of features can be performed using classifiers trained on specific taxonomic databases. |
| Normalisation(65) | Diversity analyses require an equal number of sequences in samples being compared. See also "Microbiome data". In principle, there are two different normalisation approaches:<br>- Scaling (count normalisation): There are several methods that multiply the feature table counts by fixed values or proportions, commonly a quantile of the data.<br>- Rarefaction: Random sequences are drawn from each sample, so that every sample has the same number of total counts. Samples with total counts below the set threshold (rarefaction depth) are excluded. |

## Microbiome data

When the pre-processing is complete, the data are in a feature table format, and can be combined with metadata (e.g., clinical or technical variables). The feature table has columns for each sample, and rows for every feature (ASV, OTU or taxon).

Of note, there will be different number of sequences in samples. This is due to differential efficiency of the sequencing process, and does not reflect true biological variation. Importantly, marker gene sequencing does not provide quantitative data. When looking at the features of samples, we do not know the real abundance of each feature, only the proportion of that feature in relation to the sum of features in that sample, or, in other words, the *relative abundance*. That all abundances are relative makes the data *compositional*. Compositionality complicates analysis and interpretation of data, since abundances cannot be compared directly between samples (66). For instance, if there is 1/2 *Streptococcus* and 1/2 *Prevotella* in sample 1 and 2/3 *Streptococcus* and 1/3 *Prevotella* and in sample 2, the differences between the samples could be caused by a greater bacterial load of *Streptococcus* or a lesser bacterial load of *Prevotella* in sample 2. However, the difference could also result from bacterial load of both taxa being greater in sample 2 than in sample 1, but with different proportions. Or they could both be lesser. This example is illustrated in Figure 2. The perceived difference could be caused by a combination of more or less of any of the taxa. Usually, the picture is complicated by the data consisting of not just two, but several features, all influencing the relative abundance of one another.

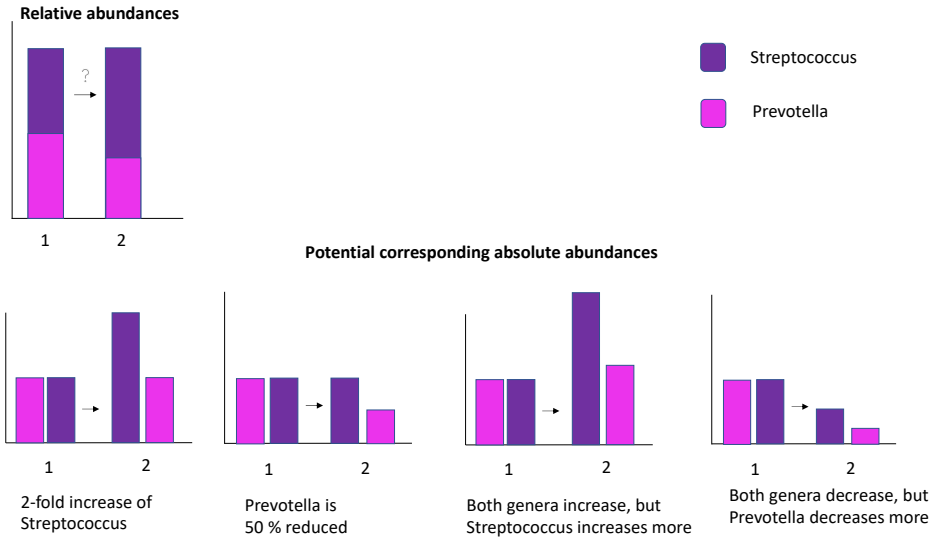


**Figure 1. Illustration of four scenarios of absolute abundances that correspond to the relative abundances of *Streptococcus* and *Prevotella* in samples 1 and 2, with explanations.**

Another common phenomenon of microbiome data is *zero-inflation*, or *sparsity*. This refers to the very large number of zeros in the feature table, which could be caused by both under-sampling and true biological differences. Both compositionality and sparsity pose challenges, disallowing use of classical statistical tests and complicates interpretation of microbiome analysis (67).

## Microbiome analysis

Analysis and presentation of microbiome data often encompass descriptions of taxonomy, results from diversity analyses and differential abundance testing. The taxonomy gives information on the names of the specific organisms that have been identified. Diversity analysis can provide information about how rich or diverse each sample is (within-sample-diversity, alpha diversity) or about how different samples are from one another (between-sample-diversity, beta diversity). There are several different ways of measuring alpha and beta diversity (diversity metrices) (68). The

diversity metrices used in this thesis are presented in the methods chapter. Differential abundance tests (DA-tests) are statistical tests developed for identification of features that significantly differ in abundance (or is otherwise differentially expressed) between groups of interest (69). Due to the challenges related to compositionality and sparsity, standard statistical tests are not applicable (67). The DA-tests used in this thesis are described in the methods chapter.

## The airway microbiome

The lower airway microbiome has not been as thoroughly studied as other human microbiomes such as those of the gut, mouth, vagina and skin. This is, in part, due to the lungs being less accessible for sampling, but likely also because they, for a long time, were not considered a site of particular interest. The healthy lungs have historically been considered to be free from bacteria. The notion of lung sterility was expressed in a paper in New England Journal of Medicine as late as in 2008 (70), and in lectures for medical students at University of Bergen as late as in 2018 (personal experience).

In 2013, at the time when data collection for the MicroCOPD study started, it had been recognised that even healthy lungs do contain microorganisms (71), that the microbiota of the lungs is different from oral microbiota, and that microbiota could show regional differences within the lung (72). In studies of healthy bacterial communities (71-73), the most common consistently observed phyla were Bacteroidetes, Firmicutes and Proteobacteria, and dominant genera included *Prevotella, Veillonella, Streptococcus* and *Pseudomonas.* However, the same taxonomic groups were seen as dominant in COPD and other disease states (71, 72, 74-76), and there seemed to be a focus on reporting alterations in the relative abundances of bacteria. In one study, Proteobacteria was found to be increased in asthma and COPD compared to healthy controls (71). Fungal microbiota was found to differ between health and disease states, but studies were few (77, 78). The literature was inconclusive with regard to diversity analysis in diseased and healthy airways. In

COPD, the diversity was reported to be increased (74), decreased (72) and equal to that of healthy individuals (75). An overview of the literature on the airway microbiota of COPD in relation to exacerbations prior to the publication of paper III is presented in Table 3.

**Table 3: Literature table depicting papers on airway microbiota of COPD in relation to exacerbations published prior to the publication of paper III.**

| Ref | 1st author | Year | Title | Number of COPD patients | Sample type | Design and outcome | Main results |
|---|---|---|---|---|---|---|---|
| (79) | Huang | 2014 | Airway microbiome dynamics in exacerbations of chronic obstructive pulmonary disease | 12 | SS | Investigated the dynamics of the airway bacterial microbiome before, at the onset of, and after an exacerbation. | Shifts in the abundance (≥2-fold) of many taxa at exacerbation and after treatment. Microbiota members that were increased at exacerbation were primarily of the Proteobacteria phylum, including nontypical COPD pathogens. Changes in the bacterial composition after treatment for an exacerbation differed significantly among prescribed therapy regimens. Treatment with antibiotics alone primarily decreased the abundance of Proteobacteria, with the prolonged suppression of some microbiota members being observed. In contrast, treatment with corticosteroids alone led to enrichment for Proteobacteria and members of other phyla. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| (80) | Millares | 2015 | Functional Metagenomics of the Bronchial Microbiome in COPD | 8 | S | Microbiota analysis in COPD patients with FEV1<50% pred >= 3 exacerbations in the previous year in stability and exacerbation states to identify the functional changes in bronchial microbiota. | *Streptococcus* and *Haemophilus* most abundant (>50% abundance together). No changes in composition at phylum or genus level from stable to exacerbation. No changes in alpha or beta diversity. |
| (81) | Wang | 2016 | Lung microbiome dynamics in COPD exacerbations. | 87 | S | Longitudinal analyses of microbiota from before, during and 6 weeks after exacerbation. | Changes in microbiota appeared to be associated with exacerbation events and indicative of specific exacerbation phenotypes. Antibiotic and steroid treatments appear to have differential effects on the lung microbiome. In particular *Haemophilus* spp. impact the overall microbial community structure. Serum and sputum biomarkers correlated with the structure and diversity of the microbiome. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| (82) | Haldar | 2017 | Microbiome balance in sputum determined by PCR stratifies COPD exacerbations and shows potential for selective use of antibiotics. | 58 | S | Longitudinal analyses of microbiota from before, at the onset of exacerbation and on day 14 and 42 after onset of exacerbation. | Reports three subgroups designated high Gammaproteobacteria (HG), high Firmicutes and balanced Gammaproteobacteria and Firmicutes, reflecting predominance or equivalence of the two target bacterial groups. The HG cluster was characterized by G:F ratios that increased at exacerbation and returned to baseline on recovery. |
| (83) | Leitao Filho | 2018 | Sputum Microbiome is A ssociated with 1-Year Mortality Followin g COPD Hospitalizations | 102 | S | Sampling of microbiota during exacerbations and longitudinal (1 year) follow-up of mortality. | Lower values of alpha diversity among non-survivors compared to survivors. Significant differences in beta diversity between groups. Survivors had a higher relative abundance of *Veillonella*. Non-survivors had a higher abundance of *Staphylococcus*. The adjusted hazard ratios for 1-year mortality increased significantly with decreasing alpha diversity. Lower survival among patients in whom sputum samples were negative for *Veillonella* |
| (84) | Jubinville | 2018 | Exacerbation induces a microbiota shift in sputa of COPD patient | 9 | IS | Paired analyses of microbiota from stable and exacerbated states. | Microbiota shifts during exacerbation were in either Proteobacteria, Firmicutes or Bacteroidetes. *Streptococcus* and *Moraxella* levels were detected during exacerbation in patients with GOLD 3. No difference in bacterial load between stable state and exacerbation. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| (85) | Mayhew | 2018 | Longitudinal profiling of the lung microbiome in the AERIS study demonstrates repeatability of bacterial and eosinophilic COPD exacerbations. | 101 | S | Paired analyses of microbiota from stable and exacerbated states. Investigation of different exacerbation phenotypes (bacterial, viral, eosinophilic). | Stability of microbiome over time was more likely to be decreased in exacerbations and within individuals with higher exacerbation frequencies. Bacterial and eosinophilic exacerbations were more likely to be repeated in subsequent exacerbations within a subject, whereas viral exacerbations were not more likely to be repeated. Association of bacterial genera, including *Haemophilus* and *Moraxella*, with disease severity, exacerbation events and bronchiectasis. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| (86) | Wang | 2018 | Sputum microbiome temporal variability and dysbiosis in chronic obstructive pulmonary disease exacerbations: an analysis of the COPDMAP study | 281 | S | Longitudinal changes in the lung microbiome and their relationship with associated COPD outcomes (phenotypes of exacerbations) with up to 2-year follow-up | The microbiome composition was similar among centres and between stable and exacerbations except for a small significant decrease of *Veillonella* at exacerbations. The abundance of *Moraxella* was negatively associated with bacterial alpha diversity. Microbiomes were distinct between exacerbations associated with bacteria versus eosinophilic airway inflammation. Dysbiosis at exacerbations, measured as significant within subject deviation of microbial composition relative to baseline, was present in 41% of exacerbations. Dysbiosis was associated with increased exacerbation severity indicated by a greater fall in forced expiratory volume in one second, forced vital capacity and a greater increase in CAT score, particularly in exacerbations with concurrent eosinophilic inflammation. There was a significant difference of temporal variability of microbial alpha and beta diversity among centres. The variation of beta diversity significantly decreased in those subjects with frequent historical exacerbations. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| (87) | Dicker | 2018 | Genetic mannose binding lectin (MBL) deficiency is associated with airway microbiota diversity and reduced exacerbation frequency in COPD. | 141 | SS | Compares microbiota in stable COPD patients with and witout genetic MBL deficiency | Patients with MBL deficiency were significantly less likely to have severe exacerbations. MBL deficiency did not affect rate of FEV1 decline or mortality. Patients with MBL deficiency had a more diverse lung microbiota and were less likely to be colonised with *Haemophilus* spp. There were lower levels of airway inflammation in patients with MBL deficiency. |
| (88) | Ren | 2018 | Transcriptionally Active Lung Microbiome and Its Association with Bacterial Biomass and Host Inflammatory Status. | 25 | BAL | Metatranscriptome and microbiota analysis in patients undergoing bronchoscopy as part of clinical management. Retrospective follow-up of exacerbations four years later. | Obtained the number of exacerbations for 21 individuals, in total 29 exacerbations. Suggest that *Streptococcus* and *Rothia* might be protective against exacerbations, and *Pseudomonas* might be harmful. |
| (36) | Pragman | 2019 | Chronic obstructive pulmonary disease upper airway microbiota alpha diversity is associated with exacerbation phenotype: a case-control observational study. | 22 | IS | Cross-sectional case control study of microbiota in frequent and infrequent exacerbators (sampling in stable state). | Frequent exacerbators had lower alpha diversity. Groups only shared 33/169 ASVs between them. Copy number not associated with phenotype. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| (89) | Wang | 2019 | Airway host-microbiome interactions in chronic obstructive pulmonary disease | 43 | S | Investigated microbiome, transcriptome and proteome in stable and exacerbated COPD (longitudinal analysis). | Reports 8.6 % *Streptococcus*. More *Moraxella* and lower alpha diversity in exacerbation samples, a trend which was also reversed at post-samples, but this was not significant in paired analyses. No significant increase in bacterial load (qPCR). In transcriptome analyses, interleukin-6 and interferon signaling pathways were upregulated in exacerbation. No differences in proteome were seen between stable and exacerbated samples. *Haemophilus* and *Moraxella* were most associated with immune response. *Megasphera* was associated with reduced expression of host inflammatory pathways. |
| (35) | Millares | 2019 | Relationship between the respiratory microbiome and the severity of airflow limitation, history of exacerbations and circulating eosinophils in COPD patients | 72 | SS | Compares microbiota profiles in stable state and studied relation to airflow limitation, previous exacerbations and blood eosinophilia. | Abundance of *Streptococcus* 1.92 %. *Pseudomonas* increased and *Treponema* decreased with increasing airflow limitation. TM7 lower in those with 1 exacerbation previous year. TM7 and Spirochetes reduced in frequent exacerbators. Increased abundances of *Pseudomonas*, *Selenomonas*, *Anaerococcus* in frequent exacerbators. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| (90) | Tangedal | 2019 | Sputum microbiota and inflammation at stable state and during exacerbations in a cohort of chronic obstructive pulmonary disease (COPD) patients. | 36 | IS | Paired analyses of microbiota from stable and exacerbated states, in addition to a case presentation with more events. | Levels of IP-10, MIG, TNF-alpha and AMPs differed between stable and exacerbation state. Of 36 sample pairs, 24 had significant differences in the most abundant genera in paired analyses, but no differentially abundant features when tested with ALDEx2. Diversity was significantly different in several individuals, but not when data was analysed on a group level. The patient case study showed longitudinal dynamics in microbiota unrelated to disease state. |
| (91) | Lòpez Caro | 2019 | Sputum Microbiome Dynamics in Chronic Obstructive Pulmonary Disease Patients during an Exacerbation Event and Post-Stabilization | 55 | S | Paired microbiota analysis of exacerbation event and post-stabilization | The study found a stable microbiome composition in the post-stabilization samples and 4 additional microbiomes in samples taken during exacerbation, 3 of which showed a marked dysbiosis by *Haemophilus*, *Pseudomonas*, and *Serratia*. The fourth exacerbation microbiome had a very similar composition to post-stabilization samples, but some pathogens such as *Moraxella* and respiratory viruses were also found. |

Abbreviations: SS; Spontaneous sputum, IS; Induced sputum; S: Sputum (type not defined, or mixed), HG; High Gammaproteobacteria, G:F; Gammabacteria:Firmicutes, MBL; mannose binding lectin, IP-10; Interferon gamma-induced protein 10, MIG; monokine induced by gamma interferon, TNF-alpha; Tumour Necrosis Factor-alpha, AMPs; Antimicrobial peptides.

Studies of the airway microbiome have presented with inconsistent findings, likely due to different methods and small sample sizes. This, and a lack of longitudinal studies, has left a series of questions unanswered. For instance: Beyond general knowledge about which high-level taxonomic groups of bacteria that are dominant, what is to be considered a normal/healthy bacterial airway microbiome? Is the composition of microbes stable over time? (How) is the microbiome altered in respiratory diseases such as COPD and asthma? What characterises fungal and viral communities in healthy and diseased airways? And (how) does the airway microbiota relate to specific aspects of disease or disease progression, such as frequency of COPD exacerbations?

These questions, among others, are central to the MicroCOPD study, which set out to be the largest one-centre study of the airway microbiome (92). Studying lower airway microbiota is, however, challenging. The main challenges are related to samples being low-biomass. Since the total burden of microorganisms is low, the samples are prone to contamination of bacteria, fungi or viruses from other sources. Presence of contaminants can cause serious errors in analysis and interpretation of data, and can be difficult to prevent, identify and account for. Contamination can stem from the laboratory processing of samples, for instance from the sample medium, the reagents in use, the researchers or research environment, or from other samples (cross-contamination) (93). Another contamination source is that of the upper airways. Sputum collection is a common airway sampling method both in microbiota research and in clinical practice. Sputum is mucus from the lower airways (spontaneously occurring, or induced for the purpose of sampling) that is coughed up. The sputum sample passes through the respiratory tract and is delivered via the high biomass oral cavity (94). Although useful for detecting pathogens in a clinical setting, sputum unlikely provides researchers with a representative picture of lung microbiota due to contamination of oral microbes. It is therefore recommended to sample the lungs directly (95). This can be done with *bronchoscopy*.

# Bronchoscopy

## History

In 1897, German otorhinolaryngologist Gustav Killian (1860-1921) used an oesophagoscope to remove a foreign body (an inhaled pork bone) from the right main bronchus of a patient. The scope used was a simple rigid tube with an 8 mm lumen, a proximal (external) light source, and a laryngoscope handle (96). The following year, after having acquired some additional experience, he presented the method as "direct bronchoscopy" (97) and has since been credited as the "father of bronchoscopy" (98). In the years prior, Przemyslaw Pieniazek had safely performed investigations in and removed objects from the lower trachea via tracheostomies. Rosenheim and Kirstein had, by accident and intent respectively, passed the oesophagoscope into the trachea. Kirstein did not dare to proceed below the level of carina. Killian became widely known for bronchoscopic foreign body removal, and patients were referred from all over Europe, and even from as far as Uruguay (99). His invention was made possible by an important discovery in the former decade; the topical anaesthetic properties of cocaine. Concurrent advances in medicine and technology contributed to the method development. Within few years, Killian performed bronchoscopy on a number of indications, including drainage of pulmonary abscesses, stenting of airways and endoluminal brachyradiotherapy (100). Bronchoscopy soon became an established medical procedure, mainly for therapeutic indications. The first textbook on bronchoscopy, written by Chevalier Jackson in 1907, presents a mortality rate of 9.6% and still states *"To-day when endoscopy has reached such a high degree of perfection (...)" (98)*. Despite its perceived perfection, the field of bronchoscopy continued to progress. Alongside innovations in other endoscopic fields, new features were added and continuously improved, including light sources, suction tubes, camera technologies and different surgical instruments (100). In the 1960s, Shigeto Ikeda developed and commercialised a thin flexible bronchoscope with glass fibre illumination (101). His invention would revolutionise bronchoscopy. In contrast to the rigid scope which limits investigations to proximal parts of the bronchial tree, Ikedas

bronchoscope has a mechanism that flexes or extends the distal end, which facilitates insertion through arched airways. Although Killian had performed his first rigid bronchoscopies with topical anaesthesia only(!) (97), sedation or general anaesthesia was usually necessary (98). In the beginning, Ikeda's bronchoscope had to be inserted through an endotracheal tube or used in combination with a rigid scope (101). As improvements was made to the instrument, bronchoscopy was soon to be less invasive, and could be performed without sedation. It was also possible to insert the new bronchoscope via the nose (102). Modern flexible bronchoscopes come with advanced video and illumination technology and an increasing number of procedures can be performed using specialised equipment through the working channel. Although rigid bronchoscopy still has a number of indications, the flexible scope can be applied for most procedures (103). Flexible bronchoscopy is described in more detail below, and is hereafter referred to simply as bronchoscopy.

## Indications

Bronchoscopy can be applied in most situations where access to the bronchi for visualisation, sampling or direct treatment is desired, and hence has a wide range of indications in diagnostics, therapy and research. Diagnostic indications include assessment of, for example, pneumonia, pulmonary infiltrates, haemoptysis, persistent cough, stridor, (potentially) malignant tissue, inhalation and burn injuries, fistulae, tracheobronchomalacia, lung transplants and foreign bodies. Therapeutic indications include for instance mucus suction, foreign body removal, endotracheal tube placement in difficult airways, tumour debulking and removal, abscess drainage, asthma refractory to medical treatment (bronchial thermoplasty), and insertion of valves, coils and stents, for instance in treating emphysema (104).
Bronchoscopy, with its diagnostic and therapeutic applications, is continuously being subject to research. However, bronchoscopy can also be performed for pure research purposes where obtaining material from the lungs is desired (see A note on research bronchoscopy). Sampling techniques commonly used in diagnostic work-ups and research are described below.

## Sampling techniques

*Brush sampling*

Protected specimen brushes (PSB), also referred to as endobronchial brushes, is a technique where specimens can be obtained using a sterile, single-use brush enclosed within a catheter sheath and protected (until advanced) by a resorbable wax-plug. The tip of the bronchoscope is placed in the desired area before the catheter sheath (with the brush inside) is advanced through the working channel. The brush can be pushed out of the sheath and rubbed against the bronchial wall, in which small parts of the endothelium will be released onto the brush. Brushes come in different lengths, widths and with different stiffness. The catheter sheath prevents contamination of the brush and protects the working channel of the bronchoscope from damage by the brush (105).

*Bronchoalveolar lavage*

Bronchoalveolar lavage (BAL) is a procedure in which a fluid (saline) is installed into a subsegment of the lung (with the bronchoscope in a wedged position, preventing fluid from escaping the segment), and thereafter recollected by suction into a sterile trap, or aspiration into a sterile syringe. Since the installed fluid is quickly distributed throughout the chosen segment, beyond the reach of the tip of the bronchoscope, BAL enables sampling that includes contents from the alveolar milieu in addition to that of the bronchial lumen. The amount of installed fluid is not standardised, but a total of 100-300 ml in aliquots of 20-60 ml is common (106, 107). Bronchoalveolar lavages of with smaller volumes can be referred to as small volume lavage (SVL). BAL sampling can be performed with different techniques. A method using a sterile catheter inside the working channel of the scope, preventing contamination of the sample, can be referred to as protected BAL (pBAL) (95). In this thesis, protected BAL is simply referred to as BAL.

*Bronchial wash*

Washing the bronchi with smaller volumes of saline can also be performed without the wedging of the scope and with smaller volumes compared to BAL (107).

*Biopsy*

Bronchoscopy can be used to obtain both endobronchial biopsies (EBB) and transbronchial biopsies (TBB) (also referred to as transbronchial lung biopsies (TBLB)). EBB can be taken using forceps in the working channel under direct visualisation of the bronchoscopist, are useful in diagnosing endobronchial lesions or in research and are associated with less bleeding compared to TBB (108). TBB are useful for sampling the lung parenchyma, and can be done blindly or guided by fluoroscopy, ultrasound or other navigation technologies (105).

**Sedation and anaesthesia**

The *British Thoracic Society guideline for diagnostic flexible bronchoscopy in adults* (BTS guideline) recommends that patients undergoing bronchoscopy should be offered intravenous sedation, preferably with the benzodiazepine midazolam, titrated to a level in which verbal contact is possible at all times (109). This light level of sedation is commonly referred to as "conscious sedation", although anaesthesiologist argue against the term since consciousness usually is altered (110). Other sedatives commonly used include Propofol (should be administered by anaesthetist) and, recommended in combination with midazolam; the short-acting opioids fentanyl and alfentanil (109). At Haukeland University Hospital, light sedation with alfentanil alone is routinely used for bronchoscopy, and additional midazolam is given to anxious patients.

Unless contraindicated, lidocaine is used for topical anaesthesia of the upper and lower airways. Lidocaine gel is recommended for anaesthesia of nostrils/nasopharynx (in the case of nasal insertion) and lidocaine spray for oropharynx prior to scope insertion. Lidocaine solution can be administered to the larynx and trachea through a

cricothyroid puncture, or directly through the bronchoscope (spray-as-you-go technique). It is recommended to note the total dosage of lidocaine given (109).

### Contraindications

The BTS guideline states only active myocardial ischemia as an absolute contraindication for bronchoscopy, in addition to situations where sedation and anaesthesia are contraindicated (109). Specific procedures performed under bronchoscopy have their own contraindications. For research bronchoscopies, more cautious contraindications may be in place (see Methods).

### Patient monitoring

The BTS guideline recommends to record the heart rate, respiratory rate, blood pressure and oxygen saturation of patients undergoing bronchoscopy, before, repeatedly during and after the procedure. In addition, it is recommended that bronchoscopy units undertake periodic audit of the efficacy, safety and patient satisfaction (109).

### A note on research bronchoscopy

Bronchoscopy can be applied as a research tool in various settings. Most often, sampling for a research purpose is conducted in patients who are already undergoing a bronchoscopy for a clinical indication (111). When this is the case, it has been argued that research bronchoscopy would be acceptable even in children (112). Bronchoscopies are also performed for scientific reasons only. For instance, bronchoscopy has been used to study lung microbiota in health and disease (71, 72, 113, 114), and drug or pollution effects on respiratory cells (115, 116). This use of bronchoscopy might be more controversial, due to the potential harm for research participants, as demonstrated in the following example: In 1996, 19-year-old Hoiyan

Wan participated as a healthy volunteer in a study on lung cell function at the University of Rochester. As part of the study, she underwent bronchoscopy. To ease her discomfort, considerable amounts of lidocaine was administered. Despite complaints of chest discomfort, she was discharged after the one-hour observation period. At home she quickly deteriorated. Upon arrival in the emergency department about three hours after bronchoscopy, she was in cardiac arrest. She died two days later. Her blood levels of lidocaine suggested that she had received more than twice the maximum safe dosage (117, 118).

## Safety of participants in clinical research

### The Helsinki declaration

Throughout the history of science, potentially harmful (and undoubtedly dangerous) experiments have been conducted on humans for the sake of new knowledge and progress in research, many of which we today would consider highly unethical (119). In 1964, The World Medical Association developed the Declaration of Helsinki, a statement of ethical principles for medical research involving human subjects. Its main purpose is to protect the rights of individuals taking part in research. The document has been revised eight times (last update in 2013), and is universally regarded an ethical norm for everyone who conducts medical research. Today, the Declaration of Helsinki includes sections concerning the obligation of informed consent, the balance between risks and benefits, the requirement for research ethics committees and research protocols, considerations of research in vulnerable persons, the use of placebo and more. Regarding risks, the declaration states:

“16. In medical practice and in medical research, most interventions involve risks and burdens.
Medical research involving human subjects may only be conducted if the importance of the objective outweighs the risks and burdens to the research subjects.

17. All medical research involving human subjects must be preceded by careful assessment of predictable risks and burdens to the individuals and groups involved in the research in comparison with foreseeable benefits to them and to other individuals or groups affected by the condition under investigation.

Measures to minimise the risks must be implemented. The risks must be continuously monitored, assessed and documented by the researcher.

18. Physicians may not be involved in a research study involving human subjects unless they are confident that the risks have been adequately assessed and can be satisfactorily managed.
When the risks are found to outweigh the potential benefits or when there is conclusive proof of definitive outcomes, physicians must assess whether to continue, modify or immediately stop the study." (120)

**Monitoring, assessing and documenting risks in research**

Although promoting the documentation of risks in research, the Helsinki declaration does not state how this should be performed.

*CONSORT – a guideline for randomised controlled trials*
Consolidated Standards of Reporting Trials (CONSORT) is a guideline for reporting and documenting methods and results from randomised controlled trials (RCTs). Many publishers require that authors of RCT publications adhere to the CONSORT statement and checklist. *Harm* is one of the items on the checklist (121). An extension regarding harm has been published, with a list of recommendations and discussions of the terms *adverse events, serious adverse events, adverse (drug) reaction, harms, active and passive surveillance of harms, safety, risk-benefit ratio, toxicity and side effects*. Not all of these terms are defined by CONSORT. Researchers are

recommended to define adverse events and preferably report events using field specific scales and validated measures (122).

*Adverse events assessment tools*

There are a number of published tools for assessment of adverse events in research and in clinical practice, see Table 4 for examples.

**Table 4: Examples of tools used to assess adverse events in research and clinical practice.**

| Tool | Description | Categorises/grades event |
|---|---|---|
| Systematic Assessment for Treatment of Emergent Events (SAFTEE) (123) | Developed for use in psychiatric clinical trials, but has been used also for other drug-related adverse events. Questionnaire for patient interviews. Very detailed: Captures onset, duration, pattern, judgement of attribution of cause, and action taken by the clinician | Up to rater to rate severity of each event. No final classification. |
| Clavien-Dindo (124) | Standardized system for the registration of surgical complications | Grade I: Any deviation from the *normal postoperative course* without need for pharmacological treatment or surgical, endoscopic, and radiological interventions Allowed regimens includes antiemetics, antipyretics, analgesics, diuretics, electrolytes, physiotherapy and bedside wound infections openings. Grade II: Requiring drugs other than such allowed for grade I complications. Grade III: Requiring surgical, endoscopic or radiological intervention. Grade IV: Life-threatening complication requiring ICU management. Grade V: Death |
| Common Terminology Criteria for Adverse Events CTCAE (125) | Developed for documenting AEs experienced by patients enrolled in oncology clinical trials. Exists in many different versions, with the addition of new explicit AEs for every revision. Also modified versions for patient self-reporting and paediatrics. | CTCAE grades are assigned based on the potential impact the AE has on clinical management, activities of daily living (ADLs), dose modifications, or medication discontinuation. Grades 1-5 for each AE. Grade 5 often = death. |

| | | |
|---|---|---|
| Global Trigger Tool (GTT) (126) | Retrospective estimation of AE rates in clinical practice. Used in medical records review. | No uniform grading/classification. |
| Systematic Monitoring of Adverse events Related to TreatmentS (SMARTS) (127) | Checklist for patients specific for detecting adverse effects of antipsychotic drugs. | No uniform grading/classification. |
| Landriel Ibanez (128) | Classification for neurosurgical complications. Can be applied in prospective and retrospective analyses. | Grade I: Non-life-threatening complication treated without invasive procedures. Grade II: Complications requiring invasive management. Grade III: life-threatening adverse events requiring ICU admission. Grade IV: Death |

AE: Adverse event. ICU: Intensive Care Unit.

Using a standardised and systematic tool can enable comparison of safety between studies and aid researchers in defining and discovering adverse events, hence reducing bias (129). Some of these tools also categorise and grade events (124, 125, 128). Categorisation contributes to data reduction which is necessary for analysis. Safety can be complicated to analyse because the data consist of many variables (many different types of adverse events/complications), have a complex structure (for instance including level of severity, timing, duration), can include longitudinal observations, and often include missing or censored data (129). Besides tools specifically developed for detecting adverse events in drug trials, no standardised tool for evaluating safety/adverse events in research has been found by this researcher, despite extensive searching.

# Objectives

The objectives of this thesis were to:

Systematically review the literature to identify bronchoscopy-related complications and discomfort, meaningful complication rates, and predictors. (Paper I)

Investigate if research bronchoscopy is less safe in subjects with than without obstructive lung disease by evaluating complications and discomfort occurring immediately, and within a week after bronchoscopy. (Paper II)

Investigate whether the compositionality of the lower airway microbiota in stable COPD predicts later exacerbation risk in a cohort study. (Paper III)

# Materials and Methods

## Systematic literature review

A systematic literature review is a type of study design which uses standardised and transparent methods for systematic searching, filtering, reviewing, evaluating and reporting information from multiple studies in order to summarise and learn from the entire available literature on the topic of interest (130).

### The systematic search

For paper I, a modified population intervention comparison outcome (PICO) form was created in order to include bronchoscopy, bronchoscopy related techniques and associated procedures, and the outcomes of interest (paper I, Table 1). The search was conducted in PubMed (Medline). Keywords were selected by combining existing thesauruses (MeSH terms) and text words after examining the existing MeSH database and the (MeSH) classification of a selection of known relevant papers. In addition, we added text words describing complications known to the authors.

The final literature search for paper I was conducted on February 8th 2016. For the discussion of this thesis, a new search was repeated on March 2nd 2021.

#### *Search filtering*

All available titles and abstracts from the search hits were read thoroughly.
Publications were excluded if they:

- Were not published in English, French or a Scandinavian language
- Described single case-reports or non-original research (letters, review articles, guidelines etc.)
- Did not involve humans (animal studies)

- Reported findings solely based on interventional procedures or specialised examination techniques
- Studied paediatric populations
- Studied severely ill patients exclusively (intubated, on mechanical ventilation, general anaesthesia, in intensive care unit)
- Clearly did not cover the topic of complications or discomfort associated with bronchoscopy. (Studies on bronchoscopes as a source of contamination were considered outside the scope of the current review.)

Remaining papers were further classified as prospective or retrospective, and whether investigation of complications and discomfort was considered an objective (primary, secondary, not formalised). We also divided articles into three groups based on the number of subjects in the study and identified studies on medication during or before bronchoscopy.

Full review was only performed on papers where complications or discomfort was a primary or secondary objective of the study, where the number of subjects exceeded 50, and where there was given a sufficient description of the sample and the sampling methods (inclusion/exclusion criteria, definition of endpoints, and data collection).

*Evaluation of papers*

Remaining papers were subsequently reviewed with respect to subtopics: death, bleeding, pneumothorax, bronchospasm, hypoxaemia, haemodynamic variations, fever and infection, health care utilisation, coughing, other respiratory symptoms and signs, and identified discomfort and pain.

# The Bergen COPD Microbiome Study (MicroCOPD)

## Study design and study population

The protocol of the Bergen COPD Microbiome Study (Short name "MicroCOPD") has been published (113). MicroCOPD is a prospective observation study conducted at the outpatient clinic at the Department of Thoracic Medicine, Haukeland University Hospital (HUH). A study pilot with eight participants was conducted between April 19th, 2012 and December 3rd, 2012 as part of protocol development. The bronchoscopies were subsequently performed between April 11th, 2013 and June 5th, 2015. The final date for collection of follow-up data was May 3rd, 2016.

Participants in the MicroCOPD study were mainly recruited from two other study populations; the Bergen COPD Cohort Study (131) and the GenKOLS study (132). In addition, MicroCOPD recruited participants from the outpatient clinic at HUH, a local pulmonology clinic (Spesialistsenteret på Straume) and the general population through local media attention.

The participants in MicroCOPD were volunteers with COPD, asthma or without known respiratory disease. The COPD and asthma diagnoses were verified by experienced pulmonologists based on spirometry (COPD: postbronchodilation forced expiratory volume in 1 second/forced vital capacity (FEV1/FVC)<0.7, according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (133)), respiratory symptoms, disease history and other diagnostic modalities including CT thorax. Participants were categorised as healthy if they did not have symptoms or lung function tests compatible with a diagnosis of airways disease.
For the sake of our participants safety, participants were excluded if they were deemed not fit for bronchoscopy. The exclusion criteria were:

- Increased bleeding risk (Double platelet inhibition, oral anticoagulant therapy, treatment with clopidogrel or ticagrelor, low molecular weight heparin

treatment; total platelet count <75*$10^9$, International Normalized Ratio >2.0; the presence of a known coagulopathy)
- Cardiac valve prosthesis
- Known severe pulmonary hypertension
- Acute coronary syndrome during the preceding 6 weeks
- Arterial $CO_2$ tension ($pCO_2$) >6.65 kPa
- Arterial $O_2$ tension ($pO_2$) <8.0 kPa or $SpO_2$ <90% despite 3 litres/minute oxygen supply through a nasal cannula
- Allergy to lidocaine or alfentanil

MicroCOPD investigated COPD during stable disease. Antibiotic treatment could influence the samples. Inclusion was therefore postponed for participants with ongoing symptoms of exacerbation or who had been treated for a COPD exacerbation or received any antibiotic treatment in the last two weeks. The form used to interview participants regarding exclusion criteria is included in the Appendices.

The reported number of participants in MicroCOPD publications has varied, due to differences in sub-studies. For instance, some publications (134, 135) include data from the pilot phase, which was not possible for the papers included in this thesis. Method papers have included data from smaller samples (95, 136).
In a non-random selection, longitudinal data collection with repeated sampling was conducted. Longitudinal follow-up of exacerbations was only relevant in participants with COPD. In paper II, the bronchoscopy safety study, the complete MicroCOPD cohort, with the exception of the eight pilot participants and two co-workers, was included in the analyses. Paper III, the exacerbation study, included participants with COPD only, and only sequencing and data from one timepoint. (Figure 2).

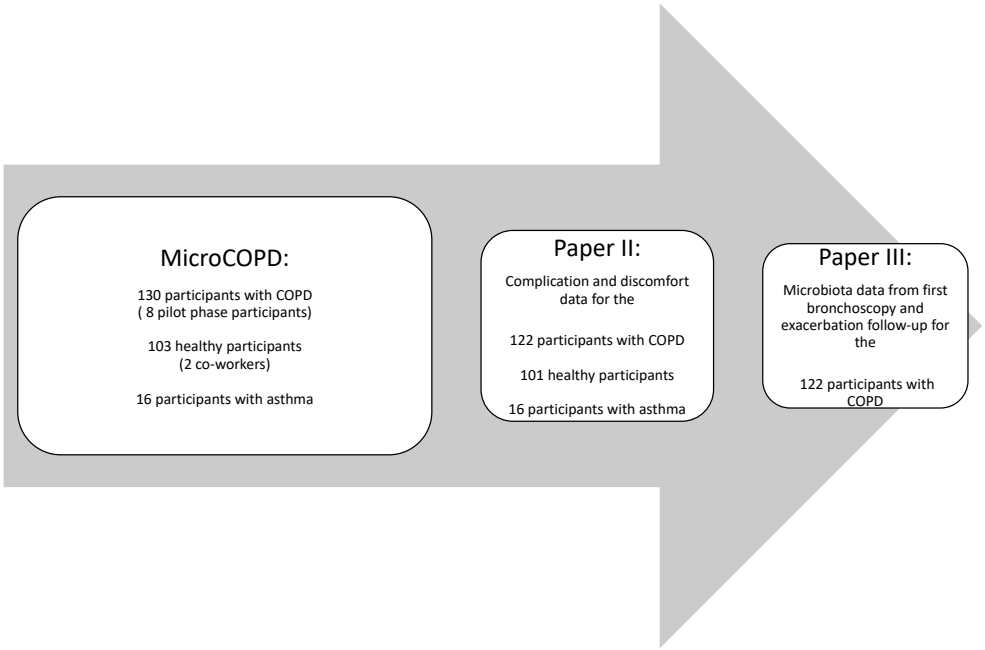


**Figure 2: Study design.** Selection of study populations within the MicroCOPD study for papers II and III.

## Pre-bronchoscopy data collection

Data collection for explanatory variables was mainly performed through a structured interview (questionnaires) and examinations (blood samples, lung function testing, blood pressure measurements, CT thorax) prior to microbial sampling. Blood gas analysis was performed in all but three participants. All participants underwent spirometry 15 minutes after inhalation of 0.4 mg salbutamol. Norwegian reference values for FEV1 and FVC were used (137).
The information was registered in a paper form (for an English translation of the form including questionnaires, see Appendix 1).

The interview included a COPD assessment test (CAT) (15), a modified Medical Research Council dyspnoea scale (mMRC) (16), the Borg scale (138), questions to identify contraindications for bronchoscopy in the study, number of exacerbations in

the preceding year, smoking history, alcohol consumption, respiratory and other co-morbidities, medication usage, time of menopause (for women), marital status, parity, education, domestic animals, motivation for study participation and dread of bronchoscopy (evaluated on a scale from 0 to 10).

**Bronchoscopy procedure**

The participant was in the supine position, and oral access was used.
Bronchoscopy was performed by one of six bronchoscopists, assisted by one of two experienced study nurses. A team of study personnel managed and prepared the samples as they were collected and partly assisted in observation and care of the participant.
In addition to salbutamol administration prior to the preceding spirometry, participants with asthma received 5 mg of nebulised salbutamol, and in some cases, per judgement of the bronchoscopist, 0.5 mg of ipratropium bromide in as well. All participants received topical anaesthesia with lidocaine by oral spray formulation (10 mg/dose) prior to the procedure and through a catheter (20 mg/mL) in the bronchoscope's working channel during bronchoscopy. All participants were offered light conscious sedation with intravenous alfentanil (0.25–1.0 mg, dosage varied per judgement of the bronchoscopist). In those who received sedation, additional alfentanil was administered during bronchoscopy if deemed necessary by the bronchoscopists. All participants received supplemental oxygen by nasal cannula, 3 L/min.
Participants were monitored by three-lead ECG and pulse oximetry throughout the procedure, and automatic non-invasive blood pressure was measured every five minutes. Most of the bronchoscopy procedures (245/323) were captured on video using the bronchoscopy camera enabling later sample location validation.

The procedure included a general inspection, protected specimen brushings, protected BAL (in participants with $FEV_1$ >30% of predicted), SVL, and in one third of bronchoscopies; endobronchial biopsies. The biopsies were taken from carinas in the right lower lobe using a sterile and disposable 1.8 mm cupped biopsy forceps after

installation of 5 mL of 0.1% epinephrine in the mucosa where the biopsies were planned.

The mean procedure duration, from passing of the vocal cords to withdrawal of the bronchoscope, was 14.2 minutes (SD 4.0).

**Microbial samples**

MicroCOPD is a large study with several methodological and clinical outcomes, which is reflected by an array of microbial samples from each participant. Sampling was not limited to the airways; a majority of participants also provided faecal samples.

The upper airways were sampled prior to bronchoscopy. All participants were asked to gargle 10 mL of Phosphate-buffered saline (PBS) for an oral wash sample (OW). Gingival (interdental) samples were included from January 14$^{th}$, 2014.

Bronchoscopy was subsequently performed to collect samples from the lower airways. Protected specimen brushes were taken from the right lower and left upper lobe, three for each site. Brushes were cut off using sterile scissors and placed in an Eppendorf tube with 1 mL of PBS. For protected BAL, two fractions each containing 50 mL of PBS were installed through a Combicath sterile catheter with a sealed tip, in the right middle lobe and aspirated through the same sterile catheter. Small volume lavage (SVL) of 20 mL PBS was instilled through the working channel in the left upper lobe and suctioned into the lavage trap.

For each participant, a sample of PBS was drawn directly from the bottle used for airway samples in that particular participant, without entering the bronchoscope or participant, to serve as a negative control sample. Pictures illustrating the sampling equipment are included in Figures 3 and 4.

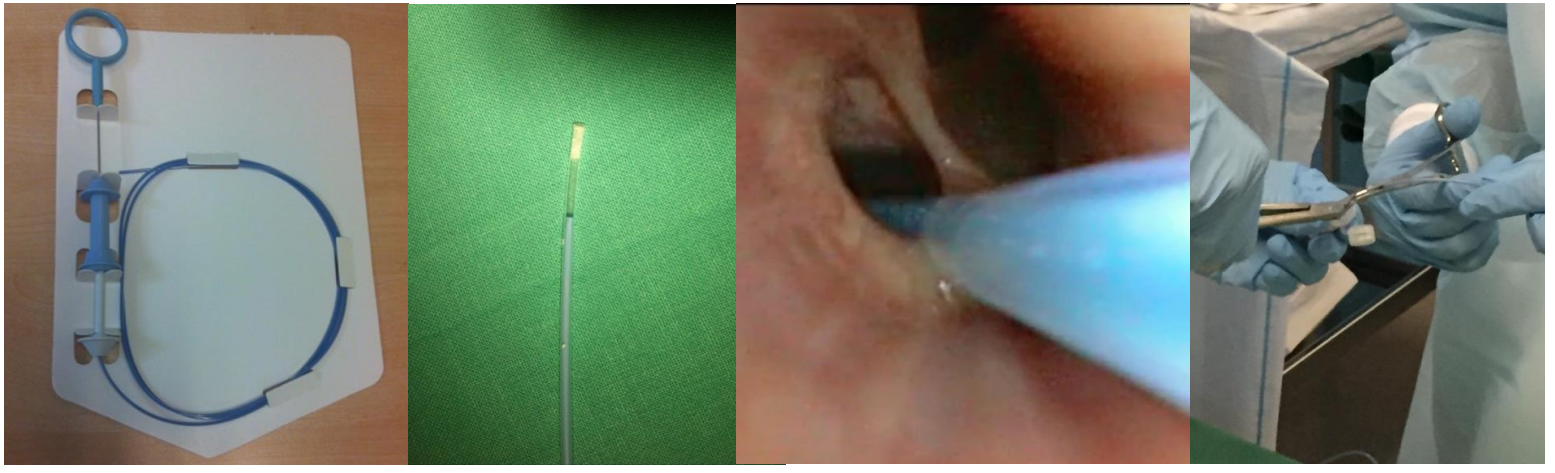

**Figure 3:** PSBs were obtained using a specialised disposable microbiology brush (ConMed, NY, USA). The brush is protected within a sheath and behind a wax plug which is expelled before sampling. After sampling, the brushes were cut off using sterile scissors and placed in Eppendorf tubes.

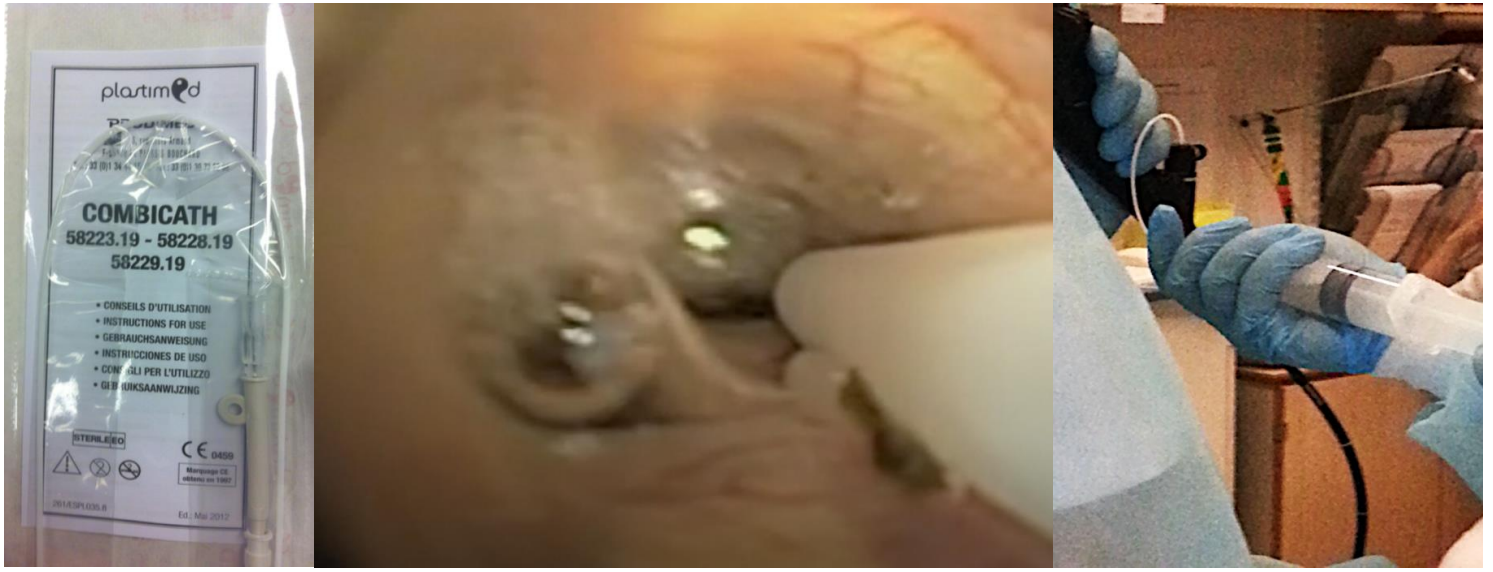


**Figure 4:** For protected BAL, two fractions each containing 50 mL of PBS were installed through a Combicath sterile catheter with a sealed tip (wax tip released in picture). BAL fluid was aspirated with manual suction through the same sterile catheter using the same sterile 50 mL syringes through which PBS buffered saline was inserted.

All airway samples and the negative control samples from the MicroCOPD cohort were included in the bioinformatic pre-processing. In paper III, the second fraction of BAL, rPSB and OW were selected for analysis. We wanted to look at both upper and lower respiratory microbiota, and more participants provided oral wash than gingiva samples. Since protected bronchoscopic sampling is found to be more suitable for distinguishing the lower airway microbiota from the oral microbiota (95), BAL and brushes were chosen over SVL. Right brushes were chosen over left brushes to cover the same geographical area as BAL. Not all first fractions of BAL were sequenced, hence the second fraction was chosen. For more details on differences between BAL and brushes, see the supplementary files of paper III.

**Measures of complications and discomfort**

Complications occurring during the procedure and a two-hour observation period was observed and recorded by the bronchoscopist and other study personnel. A complication was defined as any event that led to an unplanned intervention or premature termination of the procedure. An unplanned intervention was defined as any intervention that was not part of the prespecified bronchoscopy procedure, and deemed necessary by the bronchoscopist during or immediately after bronchoscopy. Outcomes of special interest were cough, dyspnoea, decrease in oxygen saturation, haemodynamic changes (eg, pulse/blood pressure) and bleeding. Examples of unplanned interventions included (but were not limited to) additional topical anaesthesia or sedation in the case of cough, increase in oxygen delivery in the case of desaturation, administration of (additional) epinephrine in the case of bleeding, bronchodilators in the case of dyspnoea, intravenous fluids and/or naloxone in the case of light-headedness or an observed reduction in blood pressure and antiemetics in the case of nausea. The term “severe complication” referred to situations where a participant received urgent healthcare attendance due to a threat to life or health.

Self-reported events and discomforts were recorded in structured interviews that took place on-site immediately after bronchoscopy, after the two-hour observation period

and by telephone 1 week after. The interviews were mainly conducted by one out of two medical students (including myself). Only responses from the last (1 week) interview were included in the analyses in paper II. Discomfort was graded on a 10-point scale, where 0 represented 'no discomfort' and 10 'worst discomfort imaginable'. Participants were asked to grade their dyspnoea on the Borg scale (138), which was not utilised after a quality check. Participants were also asked post-procedure about their willingness to undergo a repeated procedure, and whether they had experienced fever sensation (temperature was not measured), dyspnoea, sputum, rhinitis, wheezing chest sounds, sore throat, cough, fatigue, haemoptysis and feeling of influenza (muscle/joint ache, fever, headache, malaise). Respiratory symptom exacerbations within the following week were defined according to modified Anthonisen criteria for COPD exacerbations (44). All healthcare utilisations in the week following bronchoscopy (medication use, exacerbation treatment and hospitalisation) were recorded.

The forms used to record complications and self-reported events and discomfort were published (in English translation) as supplementary material to paper II, and are included in Appendix 1, together with other data collection forms.

**Data management and quality control of information from the data collection forms**

Handwritten information from the forms (Appendix 1) was read and interpreted by the two medical students (including the candidate) and typed into a hospital computer using the software IBM SPSS Data Collection Data Entry. The complete dataset was then transferred into a Stata file (.dta-format), and quality control with checks for inconsistencies was performed. In many cases, missing information or evident typos could be retrieved or corrected by going through the original paper form again. For participants with more than one visit, some types of information could be collected from another form. This was done with caution, and in most cases, missing

information was not possible to retrieve. The form collected large amounts of information from participants that was not used in any published analyses.

The information for variables of complications and health care utilisation following bronchoscopy was available in text only, and categorisation of events was conducted after reading through the descriptions of all events, and made into categorical variables.

**Follow-up of exacerbations**

For participants with COPD, MicroCOPD collected information on health-seeking behaviour and use of antibiotics and oral steroids in structured, quarterly telephone interviews for 12 months after the bronchoscopy. The interviews were conducted by the two study nurses[1]. Participants were also offered a follow-up examination 1–1.5 years after inclusion where exacerbation history was collected again. The original forms are presented in Appendix 2. Information from the interviews was extracted and interpreted by the candidate in the autumn of 2018. Exacerbations were self-reported, but only counted if they led to administration of antibiotics and/or oral corticosteroids, or the participant had been admitted to hospital (moderate-to-severe exacerbations). Dates reported in the telephone interviews and the physical follow-up examination were compared in order to avoid counting the same exacerbation more than once. When information on hospitalisation was contradicting, the reason for admittance was unclear (for instance hospital admittance without administration of antibiotics), or the time point for an exacerbation leading to hospitalisation was unknown, the digital hospital records were consulted.

1 When the study nurses were not present, interviews were conducted by Eli Nordeide, and in a few cases Tomas Eagan.

## Laboratory processing

The detailed protocol used for laboratory processing in the MicroCOPD study is published (139) on the repository *protocols.io*, and thus available on open access. It described all steps included to prepare the samples for sequencing. In short, bacterial DNA was extracted by enzymatic lysis and with the FastDNA Spin Kit (MP biomedicals). The V3-V4 region of the 16S rRNA gene was PCR amplified using 45 cycles and prepared for a subsequent 8-cycle index PCR step using specific primers. The samples were pooled and diluted before 2×300 cycles of paired-end sequencing was performed using reagents from the MiSeq reagent kit v3 (Illumina Inc., San Diego, CA, USA).

Samples from the MicroCOPD study was sequenced alongside mock community controls, generous donor material, other procedural controls and samples from the MicroILD study (140). The almost 2500 airway samples (including negative controls) were distributed across 30 different sequencing runs. The set-up of the different runs was planned in order to limit run batch effect and ensure that the data could be used to answer a wide range of study questions. Therefore, samples belonging to the same individual (but from different bronchoscopies) were placed on the same run, and each run included samples from both participants with and without lung disease. In addition, generous donor material (professor saliva) was distributed across 21 runs, and mock community controls were included from run 21 (9 different runs)

## Bioinformatic analyses

*Pre-processing of airway samples in MicroCOPD*

The sequencing data (airway samples and negative controls) were imported (each Illumina run separately) into QIIME 2 (141) from Casava 1.8 paired-end demultiplexed fastq format. The software Trimmomatic (142) was used to pre-trim the sequences to ensure that the all runs were treated exactly the same regarding quality requirements before the Divisive Amplicon Denoising Algorithm version 2 (DADA2)

software package (63) (via q2-dada2) was used to denoise the data. Sequences were further processed with VSEARCH(143) (via q2-vsearch) for additional chimera removal. The data was subsequently merged to one amplicon sequence variant (ASV) table.

All ASVs representing <0.005% of reads (less than 3000 reads) were removed, since they were deemed likely to represent sequencing noise (144). The ASV table was exported from QIIME 2 into R where the package Decontam (145) was used to identify and remove contaminant sequences.

The curation of data and handling of contaminants has been described in detail in the supplementary text of paper III.

*Bioinformatic analyses for paper III*

The merged decontaminated table was filtered to only include the BAL2, rPSB, OW and negative control samples from participants with COPD. Taxonomy was assigned using a classifier trained on the Human Oral Microbiome Database (HOMD) (146). All ASVs that were unassigned at the phylum level, were checked with the NCBI BLAST tool (147). These sequences were identified as nonbacterial, and therefore filtered out. Remaining ASVs were aligned with mafft (via q2-phylogeny) and a phylogenetic tree was constructed with FastTree2 (148) (via q2-phylogeny). Sequences were rarefied at a sampling depth of 1000 prior to alpha and beta diversity analyses (via q2-diversity). When applying this plug-in in QIIME 2, several distance metrices are calculated. All were initially evaluated, however only a selected few were presented in the paper. These are briefly described below:

Shannon diversity index

In 1948, Claude Shannon proposed an index that quantified the entropy in strings of text (letters) (149). It has since been widely used to calculate alpha diversity in ecology. It takes into account both the abundance and evenness of the features (for

instance ASVs) present. Shannon diversity (H) can be calculated using a natural logarithm and the proportions (p) of every feature (i)

$$H = \sum_{i=1}^{R} p_i ln(p_i).$$

Faith's phylogenetic diversity

Faith's phylogenetic diversity is an alpha diversity metric that incorporates the phylogenetic difference between the features by summarising the lengths of the phylogenetic tree branches in each sample (150).

Bray-Curtis dissimilarity

The Bray-Curtis index is a non-phylogenetic beta diversity metric (151). It is similar to the Sørensen index, but modified to take the abundances of features into account (152). Bray-Curtis index can be calculated using the number of species (n), and the relative frequencies of the species common to the samples $p_i^a \; and \; p_i^b$.

$$\frac{\sum_{i=1}^{n} \left| p_i^a - p_i^b \right|}{\sum_{i=1}^{n} (p_i^a + p_i^b)}$$

Weighted UniFrac distance

Weighted UniFrac distance is a modified version of the phylogenetic beta diversity metric unweighted UniFrac distance. Unweighted UniFrac distance is equal to the fraction of total unshared branch lengths. In weighted UniFrac distance, abundances of features are incorporated in the computation (153).

*Visualisations of microbiome data*

Taxonomy was presented in stacked taxonomic bar-plots (Paper III, Figures 2 and 3)

and in a heatmap (Paper III, Figure 4) with separate bars for each individual included to illustrate the differences between individuals as well as differences between those with and without exacerbations. For alpha diversity, box plots were used. Taxonomy plots and box plots were generated in R using data extracted from QIIME 2. The different beta diversity metrices were illustrated by principal coordinate analysis (PCoA) plots with two dimensions. In such a plot, each sample, with its sequences, is reduced to a dot. The physical distance between two dots indicates how different those two samples are. Dots that are far apart illustrate that the samples they represent are very different from one another, and two dots with close proximity have a similar content. Dots are then coloured according to the metadata variable of interest, which in the case of paper III is exacerbation frequency group, with those who had exacerbations during follow-up coloured in blue, and those without in red. If there is a true difference in the microbiota of those with and without exacerbations, one would expect to be able to see one blue and one red cluster. PCoA plots were also used extensively in the supplementary file of paper III to illustrate differences between negative control samples and study samples and to show a potential effect of run (batch effect). The PCoA plots were generated as qzv-files using QIIME 2 (Emperor plots), downloaded as images and edited in Microsoft Powerpoint.

## Statistics

The three papers are based on very different data; hence, several different statistical tools were applied.

In paper I, no statistical analysis was performed.

In paper II, bivariate analyses of explanatory and outcome variables in COPD and controls were performed using parametric (t-test, paired t-test) and non-parametric tests (Chi-squared test, Fisher's exact test, Cohen's kappa, quantile regression). For subjects undergoing more than one bronchoscopy, the outcomes of the first and second bronchoscopy were compared. Data from asthma subjects were included in the

regression models and in the overall descriptive statistics. Due to the low number of asthmatics included, no comparison between the group with asthma and the control and COPD groups was made. A logistic regression model for the dichotomous combined variable of unplanned intervention and/or premature termination of bronchoscopy (complicated procedure yes or no) and a quantile (median) regression model for the outcome of discomfort were fitted. Age and sex were included in both multivariable regression models. Additional variables were added based on bivariate effect size. Variables were kept for the final model if $p<0.1$ by a likelihood-ratio-test.

In paper III, clinical characteristics of the participants with and without exacerbations were tested using t-tests and Chi-squared tests. We tested the main outcome, which was differences in microbiota between the group with and without exacerbation using diversity metrices and tests for differential abundance (DA-tests). We tested alpha diversity difference with the Kruskal–Wallis test and beta diversity difference with the ADONIS permutation-based test.
We applied four different DA-tests. These are described in short below.

**Analysis of Composition of Microbes (ANCOM) (154)**

We performed ANCOM (version 1), which is implemented in QIIME 2, in the plugin q2-composition. ANCOM compares the log-ratios of abundances of the features (ASVs, taxa.) to detect differences in mean abundance using a linear model (close to an ANOVA test). The use of log-ratios accounts for different sequencing depths and the compositionality of the data. To deal with sparsity, ANCOM adds a pseudo-count of 1. ANCOM assumes that there are few features changing between the groups being tested. The test output is the W statistic, which gives the number of times the null hypothesis is rejected for a specific feature. In the output of the QIIME 2 plugin, the features for which the W statistic is significant are listed, and all W values are plotted in a volcano plot.

**Balance trees (gneiss) (155)**

We used balance trees for compositional data implemented in the QIIME 2 plugin q2-gneiss. The plugin first adds a pseudo-count of 1, before it creates "trees" based on correlation of features (recommended default, used in our analyses), numerical metadata or phylogeny. Gneiss calculates balances between features that can be used for further analysis. The regression used in this thesis is not supported from QIIME 2 version 2020.2 and later.

**ANOVA-like differential expression analysis 2 (ALDEx2) (156)**

ALDEx2 was performed using the QIIME 2 plugin q2-aldex2. In this method, the data are modelled as a log-ratio transformed probability distribution rather than as counts. ALDEx2 removes zeros from the data, and filters any samples with 0 reads. It takes into account the dispersion (within-condition difference) of the data, plots it against the difference and marks the differentially expressed features.

**Differential distribution analysis (DDA) (157)**

We performed DDA using the R package MicrobiomeDDA. The method tests for differences in abundance, prevalence and dispersion, and uses a zero-inflated negative binomial regression model. To account for outliers, DDA winsorises data. Specifically, DDA replaces any observation exceeding the 0.97 quantile of scaled counts for a given taxon with the 0.97 quantile of scaled counts for that taxon (157).MicrobiomeDDA normalises the data by including a scale factor to account for variable library sizes across samples. The scale factor is calculated using Geometric Mean of Pairwise Ratios (158). The R package provides a recommended built-in filtering step prior to analyses. The output is a list of differentially abundant, prevalent and dispersed features with effect sizes and adjusted and unadjusted p-values.

# Summary of papers

## Paper I:

The literature search conducted on February 8th, 2016 yielded 1707 hits, of which 1435 were excluded after screening of titles and abstracts. The most common reasons for exclusion were that the publications had no relevance to the research question (381 publications), were case studies (268 publications) or described non-original research (214 publications). Of the remaining 272 publications, 94 publications reported complications and discomfort (or equivalent) as their primary or secondary objective and included 50 subjects or more. Among these, publications that did not define outcomes, describe data collection or list criteria for inclusion and/or exclusion were excluded. In addition, four papers describing surveys of health care personnel were excluded. 45 papers remained eligible for full review.

In a study of 71 bone marrow transplant patients undergoing bronchoscopy to assess pulmonary infiltrates, 2 patients died within 24 hours after procedural bleeding following protected specimen brushing. All other studies reported a mortality rate of 0 %. Other complications rates showed considerable ranges: Bleeding (2.5-100%), desaturation (0.7-76.3%), post-procedural fever rates (2-33%), cough (4.7-86.0%), hypotension (2.9-28.9%), pneumothorax (0-4%), bronchospasm (0-12.3%) and complications in need of health care utilisation (0-31%). Variation in rates was found to be due to different study populations, different procedural aspects (such as sedation regimen) and how outcomes were defined and measured.
Measures of patient discomfort differed considerably, and results were difficult to compare between different study populations. Predictors of complications were often not presented in the reviewed articles.

## Paper II:

Complications were defined as any event requiring an unplanned intervention or early termination of bronchoscopy, during bronchoscopy or in the two-hour observation period. Complications occurred in 25.9% of participants undergoing their first bronchoscopy. Altogether 6.3% of initial bronchoscopies were aborted early. With the exception of dyspnoea, complications were not more common in participants with COPD than in healthy participants. The most frequent complications during bronchoscopy were cough (7.9%), desaturation (3.3%) and bleeding (2.9%). In the observation period, common complications were dyspnoea (4.6%, 8.2 % in COPD group, 0% in healthy group) and sedation side effects (nausea, light-headedness) (4.2%). Three participants had potentially severe complications requiring immediate healthcare attendance: Two syncopated, one experienced bronchospasm. They recovered quickly without sequelae.

There were fewer complications in participants receiving alfentanil (OR 0.27, CI 0.11 to 0.66), and more in participants with higher age (OR 1.73, CI 1.13 to 2.63).

After one week, participants with COPD reported more dyspnoea (31.4 % vs 13.9%) and increased wheezing sounds (24.8% vs 7.9%) compared to healthy individuals. For other self-reported outcomes, such as discomfort, willingness to return for a second research bronchoscopy (79.8%) sore throat/cough (55.6%) or sensation of fever (47.7%), there was no difference between the COPD and healthy group.

Significant variables associated with higher discomfort were postprocedural fever, dread of bronchoscopy and being a never-smoker.

In subjects undergoing more than one bronchoscopy, the initial bronchoscopy was often predictive for complications and postprocedural fever related to the second bronchoscopy.

## Paper III:

105 out of 122 participants with COPD had complete follow-up of exacerbations for a full year after bronchoscopic sampling. Participants who experienced one or more exacerbations within follow-up had significantly lower lung function, higher CAT score, more frequent exacerbations in the preceding year and were more often ICS users. Median time to first exacerbation was 146 days. Exacerbations were evenly distributed across seasons of the year.

In OW, BAL2 and rPSB samples, the most abundant phyla were Firmicutes, Bacteroidetes, Proteobacteria and Fusobacteria. The most abundant genera were *Streptococcus*, *Veillonella*, *Prevotella* and *Gemella*. However, the relative abundances of different taxa showed a large variation within groups and between samples. ANCOM, balance trees (gneiss) and ALDEx2 performed on BAL2 and rPSB did not identify any differentially abundant ASVs or genera between the group with and without exacerbations. Differential distribution analysis of rPSB samples identified two differentially expressed ASVs. These were classified as *Capnocytophaga gingivitis* (more abundant, less prevalent and less dispersed (adjusted p=0.011)) and *Prevotella pallens* (less abundant, more prevalent and more dispersed (adjusted p=0.041)) in the exacerbation group. The tests for differential abundance were also performed for the comparison of those with two or more exacerbations to those with zero or one, for the comparison of ICS users to non-ICS users, and to compare those with and without reported exacerbations in the preceding year. Only differential distribution analysis detected differentially expressed features. Overall, very few differences were found.

Neither alpha nor beta diversity indices differed between those with and without exacerbations in the follow-up.

# Methodological considerations

## Reliability, validity, bias and confounding

*Reliability* is the overall consistency and repeatability of a test or a measure. Different types of reliability, such as *test-retest reliability* and *inter-rater reliability* deserves mentioning. It is essential that the methods we use, for instance our questionnaires or the sequencing technology produce a similar or identical outcome if the same individual or sample is investigated in the same setting (test-retest reliability). For data collection that depend on an observer, the reliability relates to how consistent different observers are when measuring or rating the same situation (inter-rater reliability).

Reliability is an important characteristic of a measure. If the methods we use fail to produce consistent results, we cannot trust that they measure what we intend to measure. High reliability is however not sufficient, since a measure can produce consistently false results. A reliable method does not necessarily result in valid findings. Validity is the degree to which we measure what we aim to measure (159). Validity is necessary to draw meaningful conclusions from studies. *Internal validity* assesses a test or study's ability to draw conclusions regarding its study population. *External validity* is the generalisability of our findings to a larger population outside the study population. Internal validity is a prerequisite for external validity, but the external validity also depends on the relationship between the selected study population and the population of interest (160).

To produce reliable and valid results, any error arising from data collection or analysis should be avoided when possible. Avoiding random errors can be important, especially with small sample sizes. However, a more critical threat towards validity is skewness in the data that originate from systematic errors, known as *bias* (160). There are many types of bias in research, and a categorisation of biases is not entirely agreed upon (161). However, biases can broadly can be classified into two main categories:

*Selection* and *information bias*. Selection bias may refer to systematic differences between those who participate in a study and those who do not (which may affect external validity), or unintended differences between study groups that affects comparison. Information bias is also referred to as misclassification bias. An example is *recall bias,* which occurs when respondents' recollections of prior events are coloured by an outcome (160).

Confounding is sometimes referred to as a type of bias, although it is rather a confusion of effects that occurs when data is interpreted. A confounder is a variable that is associated with both the explanatory and outcome variable, leading to false assumptions about cause and effect. Confounding can often be adjusted for, but not when confounders are unknown or unmeasured. Confounding is a major problem in observational studies like the MicroCOPD study (162).

In order to obtain valuable and meaningful results, the study design must be solid, the data collection well thought-through and the analyses appropriate. In the remainder of this chapter, I draw attention to methodological issues in the current thesis.

## Study design, data collection and analysis – paper I

In literature studies, study design and data collection differ in how systematic the search strategy is. In a narrative review, a non-systematic review, there is no requirement of a systematic search, and the methodology in which the literature is searched and reviewed, is often not explained. A non-systematic review can include some systematic methodology. These reviews are often used for introducing broader research topics, although such introductions also benefit from a more systematic search. A description of a detailed search strategy helps assure the reader that important studies have not been ignored. In order to answer more specific research questions, systematic review is the recommended study design. A meta-analysis is a statistical method used to combine and analyse results from multiple studies as if it was one study. This method requires comparable study designs of included studies. A

meta-analysis particularly, but also any other literature review, is limited by the quality of the studies included (163).

**Asking the right question**

The MicroCOPD study was approved and the bronchoscopic data collection had started before paper I was planned. Research bronchoscopy of persons with and without COPD was already deemed safe based on clinical experience as well as selected sources in the literature, especially a study by Hattotuwa et al, reporting on safety of research bronchoscopy of COPD patients (164). Still, the original intention of conducting a systematic literature review was to gain a better overview of complications and discomfort associated with research bronchoscopy performed in persons with COPD as planned in the MicroCOPD study. An initial literature search limited to bronchoscopy in COPD resulted in very few hits. Research bronchoscopy is not a defined type of bronchoscopy, and could not easily be searched for. The research question was therefore widened to include other types of patients and diagnostic (non-interventional) bronchoscopy as well. The strategy was to include all potentially relevant publications in the search, and manually exclude publications according to the exclusion criteria. Such a wide research question results in inclusion of very heterogeneous papers, complicating compilation. The search could have been narrowed down to discomfort alone, to specific complications of interest, or to more specific bronchoscopic procedures or sedation regimens. Increased similarity between study methodology can enable more direct comparison of results, or even a meta-analysis. Some efforts were made to exclude studies that were too different from the MicroCOPD study in terms of patient population and bronchoscopy setting. Only looking at pre-defined complications of interest, or excluding a large number of diverse studies could, on the other hand, lead to a selection bias, potentially resulting in false low complication rates, weakening the external validity of our results.

**Searching the right places**

To reduce the workload, we searched only one database. We assumed that searching additional databases would not result in identification of additional relevant publications, as PubMed (MEDLINE) was considered the largest and most relevant medical database and non-PubMed indexed journals were regarded likely not to be relevant. This assumption deserves questioning. In a study investigating optimal database combinations for literature searches in biomedical systematic reviews, the authors estimated that 60% of published systematic reviews fail to retrieve 95% of available relevant references because they do not search important databases. They conclude that the combination of Embase, MEDLINE, Web of Science, and Google Scholar is a *minimum requirement* to guarantee adequate and efficient coverage (165).

**To MeSH or not to MeSH**

In PubMed (MEDLINE), publications are indexed using Medical Subheadings, also known as MeSH terms. MeSH terms can aid researchers by making searches more efficient and precise, enabling inclusion of publications that use alternative spellings or synonyms (166). Their usage can come at a cost of lower sensitivity if not combined with text words, as described in a study comparing subject searches to text word searches (167). In paper I, the following method description section is ambiguous:

"We used a modified Population - Intervention - Outcome comparison (PICO) form (3) (Table 1) and performed a systematic literature search in PubMed (Medline). Keywords were selected by combining existing thesauruses (MeSH terms) and text words. We performed a review of the existing MeSH database and of the (MeSH) classification of relevant papers that were already published. In addition, we added text words considered relevant to describe complications known to the authors."

Also from Table 1, which describes the modified PICO form, it is unclear which terms were used as text word and which were MeSH terms. Bronchoscopy was used as a MeSH term only, which restricted the search to papers that had received the MeSH term Bronchoscopy. Of note, the search was updated and revised for this thesis, and a more sensitive approach (including bronchoscopy as a text word) was employed, as a result of seeing that paper II would not have been identified by the original approach. Special consideration was given to publications which at the time could have been included in paper I using the revised search strategy.
This alternative search strategy identified 11 additional papers that could have been included in paper I; none of which presented important new complications or unexpected complication rates compared to those found in the original search. Additional information that should have been reviewed in paper I includes the reported safety aspects of bronchoscopy in specific populations such as patients with thrombocytopenia (168) and patients with and without pulmonary hypertension (169). This supplementary evidence from two publications does not alter any conclusion made in paper I. Also, the inclusion of these 11 papers notably would result in even wider ranges of complication rates due to added definitions and measures, which further supports the conclusion of paper I.

**Inclusion and exclusion**

To avoid exclusion of important publications and inclusion of inappropriate publications, it is necessary to specify and apply clear inclusion and exclusion criteria (170).
Language is a non-scientific exclusion criterion applied due to resource limitations, and can introduce *language bias*. The impact of English-language restrictions was assessed in a systematic review of systematic review-based meta-analyses from 2012. The authors found no evidence of a systematic language bias in conventional medicine. Yet, in order to minimise the risk of a biased summary effect, they recommend that searches should include languages other than English (LOE) when time and other resources are available (171). Given that paper I was a less specific

literature review that did not include a meta-analysis, and that some LOE were included (of note, none of them were eligible for full review) the risk of language bias is likely to be negligible.

Among the other exclusion criteria in paper I, some could undoubtedly benefit from a more detailed description. Particularly “experimental or non-standard bronchoscopy techniques” could be subject to interpretation. Even with clear definitions, it is recommended to involve more than one reviewer (170), to counteract the impact of interpretation. A common approach is to have two independent reviewers screen publications. Disagreements can be resolved in a consensus meeting or by a third reviewer. A study investigating systematic reviews showed that humans reviewers are prone to error, with false inclusion and exclusion estimated to occur at a rate of 10.76% (172). For paper I, I screened all abstracts and extracted information independently, and when in doubt, supervisor Rune Nielsen was consulted. Including a second independent reviewer could have improved the quality of paper I and increased the transparency of the research process.

**Publishing the protocol**

Another key to enhance transparency of systematic reviews is the publication of a research protocol and prospective registration of the review in a database. Health-related systematic reviews can be registered prior to information extraction in the international prospective register of systematic reviews (PROSPERO) (173). Protocols can also be considered for publication in various scientific journals. Writing and publishing a protocol could furthermore promote adherence to standards and recommendations for systematic reviews, such as the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) (174). Our protocol was neither registered nor published, and does not comply with PRISMA.

## Study design – The MicroCOPD study – papers II and III

Clinical studies can be observational, like the MicroCOPD study, or experimental. In an experimental study design, such as an RCT, the experiment is set up in a way that allows for testing of a causal relationship between one explanatory variable and outcomes of interest. Observational studies are, on the other hand, multifactorial. There is no randomisation, hence no control of other explanatory factors. This can lead to confounding, described in the beginning of this chapter. Observational studies can further be cross-sectional or longitudinal. In a cross-sectional study, the study population is investigated at one time point, which means that information for potential predictor variables and outcome variables are collected at the same time. This study design is unsuited for causality assessment. In longitudinal studies, the study population is examined with repeated measurements over time. A longitudinal study design can aid determination of the order in which events occurred, which is a prerequisite to understand causality, however in itself is not enough to establish causality. Longitudinal study designs require more time to conduct, which comes at a higher cost, and with the risk of loss to follow-up (160). The MicroCOPD study and the sub-studies of papers II and III had a mixed design, with data in part collected at one time point only and longitudinal data for selected individuals and variables.

## Data collection – The MicroCOPD study – papers II and III

In our research group, the MicroCOPD study is often referred to as the *bronchoscopy study*, since the bronchoscopic data collection clearly distinguishes it from previous studies on COPD. In this thesis, biological material from bronchoscopy was used for paper III, and the collection of safety data on this data collection method was used for paper II. Different questionnaires and forms were used to interview participants and record participant information (see Appendices 1 and 2). These variables served both as outcome and explanatory variables in paper II, and as metadata in the microbiota paper (paper III).

**Study population**

Variables of particular importance include those which separate study groups of special interest. In paper II, comparisons were often made between participants with and without COPD. And in order to maintain external validity for paper III, it was essential that all included participants had COPD. The inclusion and diagnostic criteria for COPD (described in the Introduction and Methods) are therefore important. Classifications of participants were done by experienced pulmonologists, using spirometry results, in accordance with GOLD guidelines, and performed by experienced pulmonologists. There are factors that still can influence categorization. Part of the diagnostic work-up is based on self-reported symptoms. Most participants had a smoking history. A few participants with COPD had reports of previous asthma. And, as described in the introduction, the fixed ratio criterion is debated. It is possible that we have categorised older individuals without disease as COPD, and that younger individuals with early stage of disease may have been falsely identified as healthy. We could have improved our confidence in the categories by creating a larger gap between groups, for instance by including even healthier individuals with lung function measurements above expected, or excluding GOLD 1, but this would lead to recruitment difficulties, fewer included participants (less power), and weaken the generalisability of our findings. Narrow inclusion criteria and too rigorous exclusion criteria would in general limit the external validity of our findings, as described for other studies on COPD populations (175, 176). We applied relatively few exclusion criteria, except for safety related criteria with regard to the bronchoscopy procedure.

In paper III, information on exacerbations was essential for discriminating between participant with frequent and non-frequent exacerbations. This is further discussed under Follow-up of exacerbations.

**Participant interviews**

The questionnaires used for participant interviews in the MicroCOPD study can be found in the Appendices. In part, the questionnaire form consisted of standardised and validated tests, such as mMRC, Borg scale and CAT score. Due to lack of available relevant and validated tools, the majority of questions in the structured interviews were posed using self-designed forms which we applied without extensive prior validation. Some variables were found to be error-prone in the quality assessment or in preliminary analysis. This was, for instance, the case for the Borg scale. It could have been used both as an outcome and explanatory variable in paper II, but it was clear that the participants had not understood the score. Another example of a variable that had to be discarded was self-reported information on education level, which several times varied between visits for the same individual. The structured interviews applied in the MicroCOPD study carry some risk of bias, including recall bias (since participants were asked about events in the past) and interviewer bias (since interviews were conducted by different study personnel). Uncertainty regarding the reliability of the structured interview elements could limit the internal and external validity of our findings.

**Bronchoscopy procedure**

The bronchoscopy procedure (described in the Methods) includes elements that could influence the findings in papers II and III. The procedure was standardised, and the safety of participants was of paramount importance. Therefore, BAL was only performed in individuals with sufficient lung function. This left us without BAL measurements in COPD patients in GOLD stage IV. The second most important concern, was standardisation of the sampling in order to obtain adequate microbial samples without contamination. Protected brushings and protected BAL were used, sterile scissors for cutting the brushes, sterile gloves while handling scissors and samples, and sterile PBS fluid used fresh daily are examples. However, as in most real-life studies, some compromises must be made. In order to make it possible for

some subjects to participate, sedation was only recommended, not required. This allowed some participants to drive home with their own car after the procedure. Biopsies were introduced at the time when the necessary equipment and personnel for biopsy handling became available; one year into the study. Both sedation and biopsy taking could affect perception of procedural discomforts. Biopsies were actually taken every other week, thus semi-random, but choice of sedation was by nature non-random. Importantly, there was no difference in sedation preference between participants with and without COPD.

Another point of consideration is that the MicroCOPD study had six different bronchoscopists. This could have introduced variation in technical management of procedural as well as human aspects. This could have led to systematic differences in in the quality of samples, in complication profile and in the experience of the participants. We checked the amount of BAL yield between the bronchoscopists and found small differences and microbiota profile did not differ by bronchoscopist. We did not see a difference in discomfort and complication rates between bronchoscopists. Choice of bronchoscopist was based on availability. There is no indication of systematic selection of participants based on bronchoscopist.
More emphasis could have been put on detailed monitoring and observation (see also Measures of complications and discomfort), and the procedure could have been planned in a way that would enable more systematic comparison of different safety aspects. This could, on the other hand, have drawn attention away from tasks critical to the main outcomes of the study and distort the standardisation of microbial sampling.

**Microbial samples**

Although many types of samples were available in the MicroCOPD study, we chose in paper III to limit the analysis to one oral sample (oral wash) and protected lung samples from the right lung. For the left lung, protected BAL was not performed, although small-volume washings and brushes were taken. Advantages and disadvantages of these protected sequencing techniques are described in detail in the

supplementary material of paper III. Dickson et al. have suggested that BAL of a single lobe is sufficient to examine the healthy lung microbiome (114), but it has also been reported that lungs of patients with severe COPD show more spatial bacterial variation (72). We have performed analyses comparing all sample types (95) We felt protected BAL was the superior method, and we felt the added complexity of the analyses including all samples types did not justify inclusion of samples from the left lung in paper III.

**Measures of complications and discomfort**

Safety related outcomes such as *complication* and *discomfort* are challenging to define. The terms are used in everyday language, in clinical practice and research publications, usually without explanation or clear definitions. During the review process of paper II, this researcher experienced that the connotations associated with the word *complication* made the term difficult to use. Despite complication being defined as *"any event that led to an unplanned intervention during the procedure or in the two-hour observation period, or premature termination of the procedure",* review comments included

*"I still disagree with the authors that giving additional Alfentanyl be considered a complication"*

and

*"almost every patient coughs at least once during a bronchoscopy"*

The comments above well illustrate some of the difficulties in establishing a meaningful term for this type of event in the research bronchoscopy setting. Perhaps another, less severe-sounding term, would be more accurate for some of these events. On the other hand, some events would have had significant consequences for research participants if no intervention was put in place. Operating with two different terms and

definitions is complicated. Drawing the line between common minor events and "real complications" when they all led to an unplanned intervention, would also require subjective interpretation, which we intended to avoid as far as we could.

Interventions were decided upon by the bronchoscopist, carrying a risk of observer bias. However, we could not detect any important differences between bronchoscopists. The recordings were also aided by other personnel observing and assisting the procedure and the post-procedure monitoring, which could be assumed to increase sensitivity, but further complicates interpretation of the observations. We did not attempt to investigate the inter-rater reliability in our study. In the introduction of this thesis, a table presenting some tools for assessing adverse events in research and clinical practice is shown (Table 4). None of these tools are well-suited for event evaluation in MicroCOPD, either due to differences in expected outcomes or due to a different threshold for what is to be considered a complication. In research with very standardised procedures, and where the participant gains no benefit from participation, the threshold for what is considered a complication event worthy of mentioning should be lower than in clinical practice. Having employed for instance the CTCAE tool (125) could have reduced observer bias, but would also have resulted in far lower rates, as many events relevant in this research setting would have been overseen. And importantly, even this verified tool (and others) contains elements requiring subjective evaluation. The MicroCOPD study and paper II could undoubtedly benefit from having a more detailed plan for observing complications. We could for instance have had one competent observer exclusively dedicated to reviewing all procedures. Including digital recordings of exact and continuous vital measurements could have added more transparency to our dataset. For some specific complications, such as bleeding, outcomes could have been subject to quality control using video recordings. Having bronchoscopists and other study personnel discuss what should be done in case of specific events prior to the study could have been one way of increasing the inter-rater reliability. Still, complications represent unplanned, and therefore unexpected events, which could often not have been anticipated prior to the study. The well-being and health of the participants is, and have to be, the primary concern of the

bronchoscopist. Therefore, differences are likely inevitable. This reflects the real-life situation of both the research setting that we wish to examine and that of clinical practice.

Discomfort was assessed on a scale from 0-10, one week after bronchoscopy. This measure is problematic, as different individuals can have different understanding of how the scale is to be interpreted, even though the extreme points (no discomfort/worst discomfort imaginable) were listed. Discomfort is a subjective measure, differently perceived and interpreted by different individuals. We did not attempt to define or explain any concept of discomfort to the participants. Another more studied subjective measure, closely related to discomfort, is *pain*. The numeric rating scale used to assess discomfort was developed for pain intensity assessment. Pain perception and expression can be influenced by for instance genetic, psychological and cultural factors (177). This is likely to be the case also for discomfort. Also, we do not know if participants focused on the procedure itself or the post-procedural side-effects when weighing their responses. It is plausible that those having a very unpleasant bronchoscopy would focus on the procedure itself, whereas those with more fever would have a tendency to focus on the time after bronchoscopy. Including more questions focused on specific aspects of the research participation would have been useful.

Another central measure was that of fever sensation. We did not instruct participant to measure temperature, nor provide participants with thermometers as they returned home, since the focus was on the experience of research bronchoscopy participation and because we believe transient post-bronchoscopy fever to be harmless. Patients were informed that fever was a potential side effect, which could have increased the number of reported fever episodes.

**Follow-up of exacerbations**

In exacerbation follow-up, it is important to correctly classify events, reveal all relevant events, and make sure that such events are not counted multiple times. This can be challenging even with the reasonably clear and simple definition of exacerbation that we applied. We depended on self-reported data on drug prescriptions and admissions, and did not examine the participants ourselves nor investigate events in real-time. Recall of exacerbations might be imperfect. In the SPIROMICS study, the repeatability of exacerbation recall (exacerbation in the 12 months prior) was tested in 68 study participants with COPD. The researchers reported repeatability to be low (kappa 0.42, 95% CI 0.23-0.61), but indicated that repeatability could be increased if the exacerbations were associated with antibiotic treatment or hospital admissions (178), which we employed. Their results suggest that obtaining exacerbation data retrospectively from study participants is an unreliable method (179). However, in our study, having a shorter recall period, in part cross-checking responses to information from other sources (hospital records), and only including exacerbations with associated treatment, the reliability was likely higher.

When comparing groups with few and frequent exacerbations to study a frequent exacerbator phenotype, it is essential that the threshold between frequent and infrequent exacerbations is meaningful. We chose to set the threshold at 1, which can seem arbitrary. Making a larger distinction between groups, for instance only comparing 0 to many (for instance more than 3) would limit power, as few participants had very frequent exacerbations. Of note, paper III included analysis on several cut-offs (see supplementary material of paper III), to confirm that the chosen threshold was not of importance.

# Laboratory processing – paper III

## DNA extraction

The DNA extraction step is an important source of bias in studies of microbial composition (180, 181). Since different bacteria have different cell wall structures, they usually require different DNA extraction methods, which we employed. Performing too extensive extraction might damage the DNA itself (182). In MicroCOPD, the extraction protocol was designed with the intent of securing optimal bacterial community representation. A recommended combination of three different enzymes for bacterial lysis was used (183), in order to include bacteria with potential resistance to any one enzyme. Isolated DNA was then removed before a last mechanical lysis step was applied, as a measure to avoid shearing of DNA. Even though particular thought went into the preparation of DNA, and all samples were treated identically according to the protocol, there is still a possibility of DNA extraction bias, as different bacteria are likely to have been extracted at unequal efficiencies, thereby influencing the output proportions. A way to monitor the effect of the DNA extraction step would be to include a mock community (a defined mixture of microbial cells or nucleic acid molecules created in vitro, sometimes also referred to as a *positive control*). In MicroCOPD, mock communities were included in method assessments of the PCR and sequencing protocols (184), but mock community analysis was not used to validate the DNA extraction step. The DNA extraction process can also introduce contaminant sequences (185-188). In fact, the DNA extraction kit (FastDNA Spin Kit) was found to be the main source of laboratory contamination in the MicroCOPD study (136). A discussion on the handling of contaminants in the MicroCOPD study is presented in the section Decontamination below.

## PCR

There is no standard protocol for PCR in 16S rRNA analyses. PCR conditions, including initial DNA concentration, choice of primers and DNA polymerase, amplication reaction time, reaction temperature and number of PCR cycles, vary. To compare results from studies with different protocols is challenging as we do not know the exact impact of each parameter. More importantly, these conditions have the potential to introduce error. It has for instance been shown that a higher number of PCR cycles leads to an increasing sequencing error rate and increased number of chimeras (189). However, In studies of low biomass samples, more PCR cycles are required to obtain adequate levels of DNA for sequencing coverage (190), although the signal from contaminant sequences seem to increase with the number of PCR cycles (188). Since we have studied low biomass samples, a high number of PCR cycles is arguably called for. However, while other groups studying the airway microbiota in COPD typically report 25-35 cycles (35, 79-81, 84-86, 89), we have used 45. I am not aware of any other group studying the airway microbiota of COPD that apply more than 40 PCR cycles. We studied the impact of PCR cycles in MicroCOPD and found 45 cycles to be acceptable (136). We also mitigated potential negative effects (chimera, contamination) during the bioinformatic processing (Sequence quality control and feature table construction).

## Sequencing of the 16S rRNA gene

As mentioned in the introduction, sequencing regions of the 16S rRNA gene does not provide sufficient resolution to distinguish between different species and strains of bacteria. Different regions have been shown to be biased in the bacterial taxa they are able to identify. It has been argued that the targeting of sub-regions represents a historical compromise, and that sequencing of the entire gene is not just preferrable, but also possible (61). However, even when sequencing the whole gene, there are limitations of using the 16S rRNA gene. Since the gene is essential in all bacteria, there are relatively few differences. Those genetic differences that make up the

biological differences distinguishing different bacteria from one another are largely located to other genes (191). In MicroCOPD, it would clearly be of interest to separate the COPD exacerbation related pathogen *Streptococcus pneumoniae* from typical oral commensals such as *Streptococcus Mitis*. This is not possible with 16S rRNA sequencing (192).

Our chosen sequencing platform, Illumina Miseq, have an estimated median sequencing error rate of 0.473 % (SD 0.938). The error rate is not significantly different from other, more expensive, Illumina platforms (193). Such level of error can be seen as negligible in comparison to other sources of error in the microbiota pipeline, especially those introduced by DNA extraction and PCR (194). Sequencing errors are still not random (195), and while we have attempted to mitigate other sources of bias in the laboratory processing, we have not assessed the potential of sequencing error in MicroCOPD.

**Avoiding batch effect by run**

A major challenge with performing a large microbiota study is that samples have to be distributed over multiple sequencing runs, as one plate can only fit 96 samples. The DNA extraction, PCR amplification and sequencing steps can introduce batch effects per sequencing run. Sequencing batch effects can occur due to contamination of the specific reagents on the sequencing plate, but can also be a result of well-to-well-contamination where sequences “jump” from one well to wells in close proximity on the plate (196). In MicroCOPD, the set-up of the different runs was planned in order to limit run batch effect and ensure that the data could be used to answer a wide range of study questions. By blocking samples across the plates, as explained in the methods we tried to ensure that the well-to-well contamination only added noise and not bias to our design. Particularly, we wanted all samples from the same individual to be run on the same plate to avoid batch errors when comparing sampling in participants with more than one bronchoscopy, and between sampling types within the same individuals. However, this also meant that high biomass samples (for instance OW) were processed in close proximity to low biomass samples (for instance BAL) from

the same individual. This could have caused a result where lung samples incorrectly resemble the mouth samples.
Most runs included a generous donor sample and mock communities, which assist in revealing such noise and also potentially biased runs. These positive controls were not assessed in paper III, but have been subject to analyses in other publication (136, 184), indicating that noise and run batch effects were of limited importance in MicroCOPD. However, we attempted to look for signs of batch clustering in our results (see supplementary material of paper III), and could not find evidence of such. Also, exacerbation status in the follow-up (which was unknown at the time of laboratory processing) was evenly distributed across runs. We still cannot conclude that our approach for run setup were free of errors.

**Wanted: Dead or alive**

As mentioned in the introduction, genetic methods have largely replaced culture-dependent techniques in microbiota research. Culture-independent techniques have many advantages over standard culture. Most importantly, in-vitro conditions may not allow the growth of many bacteria. For instance, only an estimated 50% of oral bacteria have been cultured (197). Proposed reasons for unculturability include lack of required nutrients in the medium, toxicity of the medium, inhibitory substances produced by other organisms present, and disruption of in vivo bacterial signalling (198). An argument against the use of more sensitive genetic methods is that they do not differentiate between living and dead bacteria, leading to an overestimation of bacterial burden and diversity. Dead bacteria do not replicate, colonise and infect, and can therefore be assumed to be of less importance. However, there is always a turnover of bacteria. Identification of DNA from dead bacteria indicates former presence of those bacteria in viable form. Also, components of non-viable bacteria have been shown to interact with different components of the immune system(199). Methods for removing DNA deriving from dead bacteria prior to sequencing have been proposed (200-202). Such methods have been applied in a few airway microbiota studies (203-207). To the best of my knowledge, no study on airway microbiota and

COPD exacerbations have attempted to sequence only live bacteria in such a manner. An alternative to removing dead bacteria is to include measures of functional output, thereby providing information on the "active" microbiota, which has been attempted in studies on COPD and exacerbations (80, 88). Not assessing the viability of bacteria is a limitation of the MicroCOPD study which is largely shared with entire microbiome field.

**Do unquantifiable findings qualify?**

As described in the introduction of this thesis and discussed in paper III, microbiota data from marker gene sequencing only provide compositional data based on relative, not absolute, abundances. In samples where we can expect the total bacterial load to be similar, this approach is justifiable. However, we do not really know if that is the case in our study. Could it be that bacterial load differs between persons with and without COPD exacerbations, and that exacerbation frequency risk is explained entirely by the number of bacteria present? Perhaps a certain threshold burden is necessary to trigger relevant immune responses as well as interactions between different members of the microbial community? Is this potential clinical impact of bacterial load taxa dependent? Quantitative measurements of bacterial load would enable interesting subgroup analysis and provide answers to some if the questions above. Statistical analysis and data interpretation would benefit from data not being compositional. There are many methods for (indirect/direct) quantification of microbiome data (208), perhaps the most common being quantitative PCR (qPCR). Quantitative PCR has its own methodological challenges and can be quite costly, especially in time employed. In MicroCOPD, qPCR was performed on a subset of samples as part of a method paper (136), but the extent of this analysis was not sufficient to evaluate whether bacterial load differed between groups investigated in paper III. Our lack of quantitative measures is arguably the most important shortcoming of paper III.

# Bioinformatic analyses – paper III

## Sequence quality control and feature table construction

Until recent years, errors in amplicon data were managed by quality filtering and clustering of sequences into separate operating taxonomic units (OTUs) if they differed by less than a set dissimilarity threshold. Typically, one would set OTU similarity to 97%. There are many OTU picking methods available (209-213). When using 97% OTUs, the rate at which errors are misinterpreted as biological variation is reduced. However, OTU picking methods underexploits the quality of modern sequencing by precluding detection of small variations that might be biologically important (214). More modern methods may be referred to as *denoising*. Denoising strategies attempt to correct sequencing errors, and group sequences into a unit where all sequences share higher similarity, sometimes referred to as 100% OTU, sub-OTU, exact sequence variant (ESV) or amplicon sequence variant (ASV) (215, 216). We chose this approach, since we believe ASVs to be more precise and reproducible (215). In our chosen pipeline, QIIME2, a denoising step resulting in an ASV table is standard. Different denoising software packages are available. We considered Deblur (64) and DADA2 (63) since they were both open source and available in the QIIME2 pipeline. Different denoising strategies have been found to produce overall similar results, although there have been found differences with regard to unweighted beta diversity metrices and alpha diversity. We chose DADA2, as DADA2 has a pooled-sample workflow where all sequences are pooled together during the denoising process. This allows for better account for batch errors across multi-run experiments. Deblur runs sample-by-sample which reduces computational requirements, but at the cost of its ability to correct batch effects (216). Of note, we chose to apply trimmomatic prior to DADA2, although this is not part of the standard workflow in the QIIME2 pipeline. We justify its use in the supplementary material of paper III. DADA2 includes a chimera removal step. As our laboratory processing might be particularly prone to chimera formation and sequencing noise, we chose to add

chimera removal with VSEARCH to the protocol and to filter out ASVs representing <0.005% of reads. These steps did not appear to remove significant amounts of sequences.

**Decontamination**

Handling contaminant sequences is essential in studies of low biomass samples. Since laboratory contamination only can be limited, not avoided, bioinformatic strategies should be applied. Decontam (145), a tool especially developed for contaminant identification and removal in marker gene sequencing studies, is probably the best available approach, as discussed in paper III. Decontam is based on two assumptions: 1) Contaminant sequences are likely to have frequencies that inversely correlate with the total DNA concentration in the sample (there is less contamination in high biomass samples). 2) Contaminant sequences are likely to have higher prevalence in negative control samples than in biological samples (145). When using Decontam, one chooses one of these assumptions or "either" as the basis for contaminant identification. We chose the prevalence-based method, which is more suitable for low biomass samples (145). The relationship between prevalence in NCS and biological samples is used to create a score for each ASV. An ASV is classified as a contaminant if the score is below a certain user-defined threshold. It is recommended to adjust the threshold according to data characteristics, which we did. We also investigated the effect of different thresholds. An advantage in our study design with regard to the prevalence based Decontam method is the large amount of NCS included. However, here lies also a potential that could not be fully exploited. Decontam does not link negative control samples to specific biological samples, but pool all samples together. It would have been interesting to see if a similar method could benefit from incorporating information connecting NCS to other samples, for instance participant ID or day of laboratory processing. A more recent tool for bioinformatic decontamination is MicroDecon (217). MicroDecon works on the assumption that all samples receive equal proportions of contamination from a common source. A "pure contaminant" feature is used as a constant for calculating how many sequences in a sample

originated from contamination. According to the developers, this tool can accurately decontaminate a single sample using a single negative control, suggesting that it could be used for matching negative control samples with their respective samples. However, it is not designed for this purpose and adaptation suited for the MicroCOPD study would require highly resource-demanding extensive manual processing and carry a risk of introducing error. Also, based on our limited knowledge of the airway microbiota composition, it would be challenging to point to an adequate constant contaminant. This constant would also differ between sets of samples. An option would be to add a known contaminant to all samples to serve as a constant, which arguably would complicate analyses.

**Diversity analysis**

We applied several different diversity metrics, which is recommended as there is no standard for which to use and interpretation is subjective (218). In our selection of metrices, we made sure to include both phylogenetic and non-phylogenetic, and both weighted and unweighted, methods. We assessed more metrics than presented in the paper, but did not consider methods not available in QIIME2, and can therefore not guarantee that choice of metric could not have influenced the results, although it is unlikely that there is a biological signal that none of our diversity metrics could uncover.

Creation of diversity metrics is complicated by differences in sequencing depth across samples. By rarefying the ASV table to a specified depth, all sample counts were randomly subsampled to the given depth (1000). Samples with a total read count lower than 1000 was consequently excluded. Higher values retain more information, but may result in a bigger sample loss. The depth was chosen based on examination of the ASV table and from alpha rarefaction plots in QIIME2, but arguably represent a subjective evaluation. Rarefaction has been criticised for discarding information and for low reproducibility (219). As touched upon in the Introduction, other normalisation methods might be worthy of consideration (65). We chose rarefaction as recommended by the QIIME2 developers, being the default option in their diversity

plugins. Since we were uncertain how this step influenced diversity analyses, we also checked this processing step by testing cumulative sum scaling (CSS) (220) in QIIME1 (221). CSS did not markedly influence the diversity results.

**Visualisations of microbiome data**

Visualisations are an important part of microbiota dissemination and should be used to aid interpretation of the data. It is important that they do not mislead readers (or the researchers).

We chose taxa bar plots with separate bars for each individual included to illustrate the taxonomic differences between individuals as well as differences between those with and without exacerbations. Others have merged taxonomy from different individuals within a group for between-group comparison (222, 223), which disguises potentially important information. Intraindividual variation may be significant, and outliers may skew the overall taxonomy.

Distance metric visualisations can be made using different dimensionality reduction tools, and different techniques may be applied to illustrate (potential) clustering between dots. We chose PCoA plots as these were readily available as part of the QIIME2 pipeline, thereby only colour coding our data points. We did not include any supervised clustering techniques (no starburst patterns or confidence eclipses), as such visualisations can cause the eye to perceive discrete clusters as stronger than they are (224, 225). A much-discussed phenomenon in ordination plots, is that of the *horseshoe effect*, which refers to a linear gradient that appears as a curve in ordination space. The horseshoe effect has been seen as a mathematical artifact requiring modification of data or disregard of results. This is not necessarily true for microbiome samples (226). Since it is not clear how or if a horseshoe effect in the ordination plot should be handled, we have not addressed the issue further. However, this effect represents a potential for misinterpretation of our data.

Alpha diversity was originally illustrated using violin plots, since these provide more information on the distribution of data, which can enrich interpretation (227). The violin plots were replaced with box plots for the publication, per reviewer request.

# Statistics – papers II and III

## Calculation of power and sample size

Statistical testing is not just what researchers do with data *after* data collection; it is also a fundamental part of planning clinical studies. A power analysis can determine the sample size needed to ensure a high probability that the study correctly rejects the null hypothesis (228). For example, we can calculate how many participants with and without COPD we need to be certain that participants with COPD experience more dyspnoea following bronchoscopy. Importantly, these calculations should be done prior to data collection and statistical testing (229). If we trust the power calculation, and use the recommended sample size, we strengthen the conclusion of the study. Another reason to calculate sample size has to do with research ethics; ideally a study should not include more participants than necessary, since research poses a potential risk to participants and cost more money (that could be better spent). How power calculations are performed depend on the outcome variable and the study group design. Elements required for sample size calculation are the desired level of power (commonly set to 0.8 or higher), significance level (commonly 0.05) and the effect size. The effect size is here the difference that needs to be detected between groups in order for it to be clinically relevant, or in other words, interesting (228). For instance, if 50% of participants with COPD and 51% of participants without COPD experienced fever following bronchoscopy, the difference in incidence would clearly not be interesting, even if statistically significant.

No sample size calculation was performed for the MicroCOPD study, because there is no consensus on what is considered a relevant effect size in microbiome studies or how it should be calculated. Commonly applied power analysis methods usually make assumptions that are not valid in the analysis of microbial communities (230). Some methods have been suggested, for instance power analysis based on pairwise distances of beta diversity metrics (like UniFrac) for PERMANOVA testing (231). This would limit the power estimation to global measures of community structure. La Rosa et al.

previously suggested a method for power analysis prior to taxonomic based testing (232), but to the extent of my knowledge, power calculation methods for commonly used differential abundance tests are not developed. Although Kelly et al. suggest ways of defining effect sizes with regard to beta diversity (231), challenges remain due to limitations in the literature. We still do not know what is a relevant difference in beta diversity between groups (or the relevance of different taxonomic distributions) in the field of airway microbiota. Also the statistical integrity of paper II is affected by the lack of a sample size calculation. The outcomes of paper II were secondary to the overall outcomes of the MicroCOPD study, and not suited for sample size calculation.

**Multiple testing**

In microbiome studies, which are typically explorative in their nature, there are very many variables tested (perhaps without a well-considered hypothesis). For instance, each feature (for instance ASV) might be tested as a separate variable. The issue of multiple testing is distinctly more pronounced in microbiome studies than in more traditional observational studies. Still, it is worth considering the impact of multiple testing also in paper II. There were many comparisons made between participants with and without COPD (41 outcome variables). This relatively high number of hypothesis tests carries a substantial risk of false positive test results (233). We did not perform any correction for multiple testing in paper II, although it would probably have been justified. One option would be to adjust the significance threshold. Of note, too rigorous correction of multiple testing may lead to false negative results (233). In paper III we did not perform correction for multiple testing where this was not included by default by tests. However, with no positive test results, this was also not called for.

**Differential abundance testing**

A plethora of methods for differential abundance testing exist. Different tests have been shown to produce a highly variable number of significant ASVs within the same

microbiome datasets (234). There is no consensus on which test one should use. The reasons for choosing these exact tests (ANCOM1, ALDEx2, gneiss and MicrobiomeDDA) and this exact number of tests(four) in paper III, are arguable somewhat arbitrary. For instance, the first version of ANCOM was chosen over succeeding versions (ANCOM version 2 and ANCOM-BC), as it was readily available as a plugin in QIIME2. MicrobiomeDDA is not commonly used in the field, but the test's ability to test for dispersion and distribution was appealing. However, all the tests have strategies to address the compositionality of the data, and they have built-in mechanisms that adjust for multiple testing or reduce the false discovery rate. The different tests do however have different assumptions, and there is no simple way to figure out if the assumptions have been violated. One cannot judge from the test result if the test was adequate. Both negative and positive findings could simply be a result of the test chosen. By presenting (a comparison) of multiple tests, we strengthen the integrity of the results. Recently it has been recommended to use a consensus approach based on multiple differential abundance tests to help ensure robust biological interpretations (234).

Most of the limitations discussed in this thesis are widely shared with other studies on the same topics. Important limitations are related to a missing standard or consensus, both when it comes to defining complications and discomfort and in the microbiome field. The MicroCOPD study importantly incorporated several method improvements which strengthens the integrity of our results. The results of this thesis are discussed in the next chapter.

# Discussion of results

## Complications and discomfort of bronchoscopy – papers I and II

Paper I was a literature review on this topic, which is closely related to that of paper II. The results of these papers are discussed in relation to one another and to other literature sources, mainly identified in the revised and updated literature search (see Methodological considerations, To MeSH or not to MeSH).

### Bronchoscopy complications and their rates

The complication rates for specific events in the MicroCOPD study (paper II) were all within the ranges of the complication rates found in the literature review (paper I), with the exception of post-procedural fever which in paper II was a self-reported discomfort measure (discussed in the next section). Given the wide ranges of complication rates in the literature, this was to be expected.

Severe complications are rare. As described in the introduction, the procedure-related mortality has improved much since the early days of bronchoscopy, but still sporadically occur, even in healthy individuals. Two papers in the updated and revised search both reported a mortality rate of 0.2% (235, 236). Death was reported in one patient who developed AECOPD following bronchoscopy (235). This finding is particularly interesting with regard to our study population, and emphasises the value of providing the participants with the physician's phone number, questioning participants about respiratory symptoms and illness after one week, as well as our specific follow-up of exacerbations in the MicroCOPD study. In another paper, death followed massive bleeding in a patient where warfarin treatment was recently initiated, but not disclosed to the bronchoscopist (236). In the MicroCOPD study, anticoagulant therapy was thoroughly enquired. More importantly, the biopsy technique used to collect biopsies in MicroCOPD has a much lower bleeding risk than the transbronchial needle aspiration performed in the mentioned study. Another paper reported no deaths caused by bleeding, but other causes of death were not mentioned, which leaves the

overall mortality rate unknown (237). A mortality rate between 0 (in most studies) and 3% (in a single study of somewhat selected individuals) is imprecise, and unacceptably high. A mortality rate of 0.2% is too high for bronchoscopy to be considered a safe procedure in research, but not all bronchoscopies are similar, with some procedures carrying a higher risk. The chance of inducing bronchospasm is possibly related to length of procedure, but can in principle always occur. In MicroCOPD, all patients received beta$_2$ agonist inhalation prior to the bronchoscopy. And, in the bronchoscopy lab where the study was performed a bilevel positive airway pressure machine is always available for emergency use after the procedure if needed. This was never the case in MicroCOPD.

The true mortality rate of bronchoscopy is lower than the studies suggest, and larger populations (for instance with the help of a meta-analysis) are necessary to estimate the accurate rate. Instead of only including studies reporting on mortality as a potential outcome, we could have assumed that other studies on bronchoscopy safety would have reported on death if it occurred. Paper II did not report on death, but I believe it is clear to the reader that no participant died. We also did not specifically report on another well-known rare complication; pneumothorax. In paper I, pneumothorax occurred at a rate between 0 and 4%. In the updated literature search, the range is between 0 (238-240) and 7.5% (241). There was no known pneumothorax in the MicroCOPD study, which was also expected given the minimally invasive sampling methods used. However, we did not screen for pneumothorax specifically.

Other, less severe complications had wide ranges in rates in paper I. The rates were expanded by the new literature search that was done for this thesis. Rates should be compared for each complication type separately in more homogenous populations with regard to specific bronchoscopic techniques and sedation regimens. Some predictors of complications are discussed below (Predictors of complications and discomfort). The types of complications in the updated literature search were the same as to those identified by papers I and II, with the addition of epistaxis (242-244) and vomiting (242, 243, 245, 246), reported with rates ranges 0.2-12.9% (243, 244) and 1.0-2.1% (242, 245), respectively. Use of oral access and strict fasting prior to the procedure might explain why these events did not occur in the MicroCOPD study. Retching

during bronchoscopy and nausea following the procedure was not uncommon. Prompt administration of antiemetics might have eliminated vomiting as a complication. The updated literature also included two papers reporting on recovery time/profile as a safety measure (247, 248). There were three papers that used the systematic complication assessment tool CTCAE (249-251).

**Self-reported measures: Fever and discomfort**

Paper II relied on a self-reported sensation of fever, which likely explains the much higher fever incidence in paper II compared to the fever rates found in paper I (2-33%) and in the updated and revised literature search (0-16.2%) (240, 250). Interestingly, Crawford et al. also reported on fever-like symptoms without temperature change, and reported a rate as low as 0.7% (252). Another study reported separate rates for fever (4.3%) and chills (3.5%) (246), the latter necessarily being self-reported. There is a possibility that our high fever rate is influenced by other, unknown factors, as well. As argued in Methodological considerations (Measures of complications and discomfort), continuous discomfort measures are difficult to compare across studies, and are mainly used for comparison between study groups for papers included in paper I. The most comparable sign of acceptance/tolerance (or oppositely discomfort) of the procedure is "willingness to return", which was 79.8% in MicroCOPD, between 55.4 and 96.3% in paper I, and between 49.1% (study subgroup) (253) and 96.8% (254) in the updated literature search. Paper I did not reveal pain as a typical complaint of bronchoscopy, and queries about pain was only included in more general questions like "have you (…) experienced flu symptoms (fever, muscle/joint ache, headache, reduced general condition)?", which cannot be used to assess pain specifically. In a Polish bronchoscopy study, 25% of patients reported post-procedural pain (246). Discomfort is difficult to quantify and compare between studies. Perhaps the focus of paper I could have been directed towards qualitative studies for discomfort related outcomes. We could also have applied qualitative methods in MicroCOPD to gain a deeper understanding of the discomfort related numbers presented in paper II, but including all 249 participants would not be feasible, and deciding upon a selection would require

caution. A qualitative study addressed experiences of bronchoscopy with conscious sedation and analgesia in high-risk respiratory patients (255). The authors concluded that patients often are conscious during the procedure and that their experience may be uncomfortable and distressing. Despite negative experiences, participants were accepting of their experience, considering it a "necessary evil" to obtain a diagnosis (255). This conclusion is neither very informative nor surprising, and adds little to my impression from the MicroCOPD study. The COPD patients in the qualitative study underwent diagnostic bronchoscopy for investigations into potentially malignant disease. Whether research bronchoscopies can be compared to the "necessary evil" of obtaining a cancer diagnosis is quite questionable, and highly depends on the perceived scientific impact of the obtained samples and the altruistic motives of research participants. Motivations for undergoing research bronchoscopy has been investigated by our group (134, 256).

**Predictors of complications and discomfort**

Knowing what causes (and increases rates of) complications would be useful. Predictors of complications could serve as contraindications for research bronchoscopy. With the exception of bleeding predictors, paper I did not identify predictors for complications and discomfort in the existing literature. In paper II, potential predictors were analysed using regression analyses. Higher age and not having received light sedation were variables predictive for complication. Having experienced fever, being a current smoker, and dread of the procedure were predictive for reports of higher discomfort. In the updated literature search, predictors for combined safety outcomes, similar to the combined outcome in paper II, were reported on. One study of hematopoietic stem cell recipients found an increased odds of a bronchoscopy related complication if TBB was performed and where the patient had undergone myeloablative conditioning, but found no association between the number of biopsies and a complication (239). Another study reported BAL to be significantly associated with complications (257).

Although testing for differences between participants with and without COPD for a

number of outcomes, we did not attempt to look for predictors of specific events like for instance bleeding, due to low incidence and power.

The literature, however, can assist our understanding of bleeding predictors further. Bleeding rates varied in studies with comparable definitions and study designs, indicating that risk of bleeding also depends, and could potentially be stratified, on type of bronchoscopic intervention or other factors. In paper I, superior vena cava syndrome (SVC) and addition of EBB and TBB to TBNA were reported to be predictors of bleeding. The finding of SVC as a predictor of bleeding was supported by Muthu et al., who retrospectively identified predictors of severe bleeding during endobronchial biopsies. The study also proposed central airway lesion and assessed tumour vascularisation degree as predictors of more severe bleeding, whereas forceps type did not predict bleeding severity (235). Another retrospective study of 80 participants suggested that bleeding risk could differ depending on which lobe the biopsy was taken from, and reported bleeding to be more if biopsies were collected using alligator forceps compared to cupped forceps, although this difference did not reach statistical significance (241). Hou et al. reported no difference in bleeding rates between groups that had biopsies taken before or after bronchial brushings (258). Bleeding risk was not reported to be different among different groups of patients. Grendelmeier et al. reported, like paper II, no difference in bleeding risk between persons with and without COPD (254). Bleeding was also similar in COPD patients with and without pulmonary hypertension (169). One study even reported that bronchoscopy safely could be performed in patients with thrombocytopenia, with platelet counts as low as $30*10^9$/L (168). In MicroCOPD, thrombocytopenia (tpc $<75*10^9$/L) was a contraindication for bronchoscopy. In light of this information, and given the minimal invasiveness of the procedure, perhaps a normal platelet count is not required for safe research bronchoscopy. The relevance to MicroCOPD is minimal, as no participant was excluded based on this criterion. Another contraindication in MicroCOPD was known severe pulmonary hypertension (not defined or evaluated in MicroCOPD, but left to the judgement of the bronchoscopist who had access to the participants' electronic medical journal). A study reported that diagnostic

bronchoscopy could be safely performed in patients with mild, moderate and severe pulmonary hypertension, although these patients experienced more hypoxaemia (169). Paper I and the updated literature search did not identify studies on complication and discomfort in repeated bronchoscopies besides from paper II. We report that a first research bronchoscopy was predictive for complications, as well as self-reported fever. This finding is plausible, but since the safety of repeated bronchoscopies is only studied in the MicroCOPD study, its implication is uncertain.

**Bronchoscopy in COPD**

Paper I could not conclude on the safety of bronchoscopy in specific patient populations, such as COPD. Paper II concluded that research bronchoscopy performed in the MicroCOPD study was safe in both participants with and without COPD. That clinical bronchoscopy safely can be performed in COPD is supported by two studies from 2017 (254, 257). In 2019, Wells et al. published a paper on the safety and tolerability of research bronchoscopy of participants with and without COPD in the prospective SPIROMICS study (259), which also was a microbiota study sampling the lungs of participants with and without COPD using bronchoscopy (260).
Clinically relevant adverse events in the SPIROMICS study were adverse events that required the following interventions: Pharmacologic treatment (i.e., administration of bronchodilators, antibiotics, and/or corticosteroids), a new supplemental oxygen therapy requirement after bronchoscopy, or hospitalisation for any reason. It is not clear if this definition was always used, as the paper seems to distinguish between adverse events and adverse events requiring intervention. Of note, this distinction can be confusing also in MicroCOPD, since tables in paper II included all events. In SPIROMICS, adverse events requiring intervention occurred in 14 of 208 (6.7%) of bronchoscopies, which is considerably less frequent than in the MicroCOPD study. Importantly, adverse events were more common in participants with COPD (defined as $FEV_1/FVC < 0.7$) than in those without COPD (11.8% vs. 2.6%). Conflicting findings can result from differences between bronchoscopists and study investigators in defining (the need for) interventions, reflected by the difference in the overall

complication rate. The criteria might have been stricter in the SPIROMICS study. Specifically, there were no patients requiring reversal agents for anaesthesia or intravenous fluid boluses for hypotension. Early termination of bronchoscopy was not mentioned. Also, age is a potential confounder in the SPIROMICS study; the COPD group in SPIROMICS was seven years older than the non-COPD group. In MicroCOPD, higher age was associated with more complications.

## Airway microbiota and exacerbations of COPD – paper III

### Exacerbations and participant characteristics

The comparison of microbiota between those having frequent exacerbations and those who did not, depended on separation of participants into groups in the follow-up and on participants not being lost to follow-up in a systematic manner. We could not have anticipated the occurrence of sufficient exacerbations and the separation of groups in the study. In the beforementioned SPIROMICS study, a potential association between lung microbiota and prospective exacerbation events was not possible to assess due to limited number of events (260).

Table 1 of paper III shows MicroCOPD participant characteristics at baseline by exacerbation status during follow-up. Participants who experienced one or more exacerbations in the follow-up had significantly lower lung function, higher symptom score, more frequent exacerbations in the preceding year and more use of inhaled corticosteroids (ICSs) compared to those who did not. These differences were expected and in line with known predictors of exacerbations (37), supporting our phenotype classification. A total of 17 participants had to be taken out of our analyses due to missing exacerbation status in the follow-up, resulting in less power. We do not believe that these missing participants led to bias in our results. Although one could speculate that these individuals were unavailable for follow-up due to disease, this is unlikely. Most of them had some initial follow-up without exacerbations. If reporting on frequent exacerbations early in the follow-up, they could have been included

without complete follow-up. Table 1 in paper III indicates that those with unknown exacerbation status might also be healthier than the other groups, with regard to lung function, symptom score, exacerbation frequency in previous year and ICS usage. We did not perform extensive microbiota analyses on the participants with missing exacerbation status. However, there were no obvious difference in taxonomy, as illustrated in Figures 5 and 6.

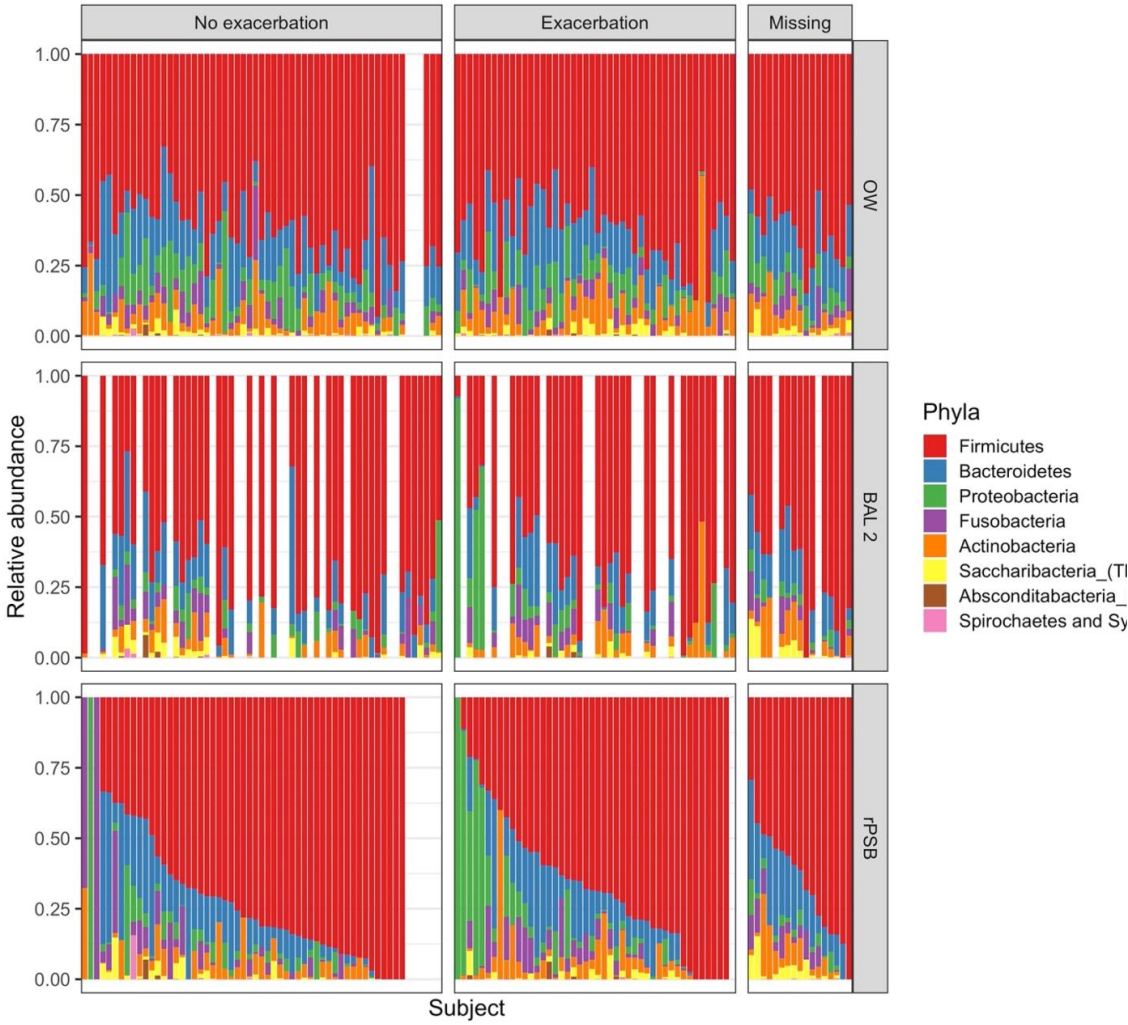


**Figure 5: Bacterial taxonomy at the phylum level in participants with COPD with and without exacerbation and participants with missing status during follow-up.** Taxonomic groups in the legend are sorted in decreasing order based on the average relative frequency of that group in all samples. Each bar represents one participant, ordered in the same position horizontally for all three sample types according to relative abundance of Firmicutes in rPSB samples. OW: oral wash; BAL2: second fraction of bronchoalveolar lavage; rPSB: right protected specimen brushes.

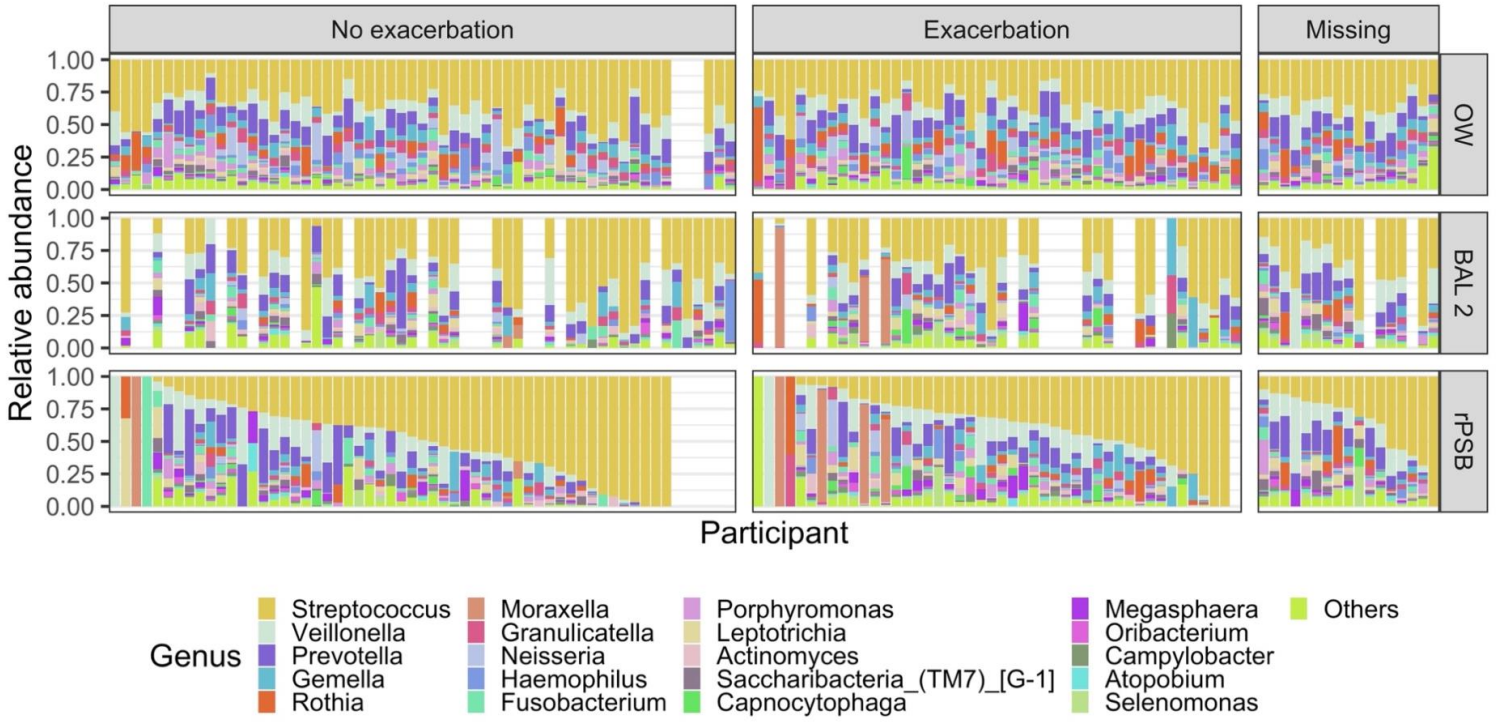


**Figure 6. Bacterial taxonomy at the genus level in participants with COPD with and without exacerbation during follow-up.** Taxonomic groups in the legend are sorted in decreasing order based on the average relative frequency of that group in all samples. Each bar represents one participant, ordered in the same position horizontally for all three sample types according to relative abundance of *Streptococcus* in rPSB samples. OW: oral wash; BAL2: second fraction of bronchoalveolar lavage; rPSB: right protected specimen brushes.

## Taxonomy and differential abundance testing

Unlike previous (35, 36, 88) and more recent (261-263) reports, paper III did not identify any association between taxonomic distribution in stable COPD lung samples and exacerbation frequency. Below I discuss three taxa of particular interest.

### *Streptococcus*

As discussed in paper III, Ren et al. suggested a protective effect of *Streptococcus* (88). Visual inspection of figure 3 in paper III (or figure Y above) could lead to this conclusion as well, but importantly, none of the four differential abundance tests we applied found *Streptococcus* to be differentially abundant. Contrary to this, a longitudinal multicentre study investigating spontaneous sputum from 200 individuals with COPD reported that presence or higher abundances of *Streptococcus* was correlated with frequent exacerbations (261). Also, from an observational study of 253 stable COPD patients, Dicker et al. reported that more sputum microbiomes characterized by *Streptococcus* dysbiosis (defined as *Streptococcus* making up >40% of OTUs) belonged to GOLD group D (263). Another recent cross-sectional study investigated induced sputum microbiota from 78 COPD patients classified into categories of low-risk exacerbators (< 2 moderate exacerbations and no severe exacerbations per year, n = 60) and high-risk exacerbators (≥ 2 moderate or severe exacerbations or ≥ 1 hospitalizations for COPD exacerbation, n = 18) based on retrospective exacerbation data. In this study, the Mann-Whitney test identified *less Streptococcus* in the high-risk exacerbators, but this finding was not significant after correction for multiple testing (Bonferroni) (262).

The inconsistent effects reported for *Streptococcus* could be a result of the different methods applied. Results are difficult to compare across studies with different sample types, laboratory handling, bioinformatic processing, and choice of statistical tests, as already discussed. Inconsistencies in the literature support the finding that *Streptococcus* is *not* differentially abundant or expressed based on exacerbator phenotype. Of course, a negative, positive, or no correlation between *Streptococcus* and the frequent exacerbator phenotype could all represent true biological results for

different patient populations. Importantly, different results for *Streptococcus* could simply be an effect of low resolution of 16S rRNA gene sequencing for this specific genus. The protective effect seen by Ren et al. could be driven by a specific species not present in other studies, and that *Streptococcus* was correlated with frequent exacerbations could potentially be caused by a greater relative abundance of *S. pneumoniae* or another pathogenic species in other studies.

*Pseudomonas*

Two papers published prior to paper III point to an association between *Pseudomonas* and exacerbation frequency (35, 88). A similar association has been presented also in newer publications (261-263) (although in two papers the actual testing has been performed at order (262) and phylum (263) level). Contrary to that of *Streptococcus*, the effect of *Pseudomonas* does not show conflicting directions when mentioned in the literature. In paper III, *Pseudomonas* was identified as a contaminant and removed from analyses. In unfiltered data, *Pseudomonas* was found in high abundances in samples both from participants with and without exacerbations in the follow-up, as illustrated in Figure 7.

*Moraxella*

This known exacerbation-related pathogen was present in very high abundances in five participants, four of which were in the frequent exacerbation group. Similar findings were presented by Pragman et al. (36).

Although the presentation of *Moraxella* could not be a predictor for exacerbations in the group as a whole, we speculated that this assumed microbiota alteration could have a clinical impact on these selected individuals. Stable state *Moraxella* has been reported to be associated with frequent exacerbations by others (261).

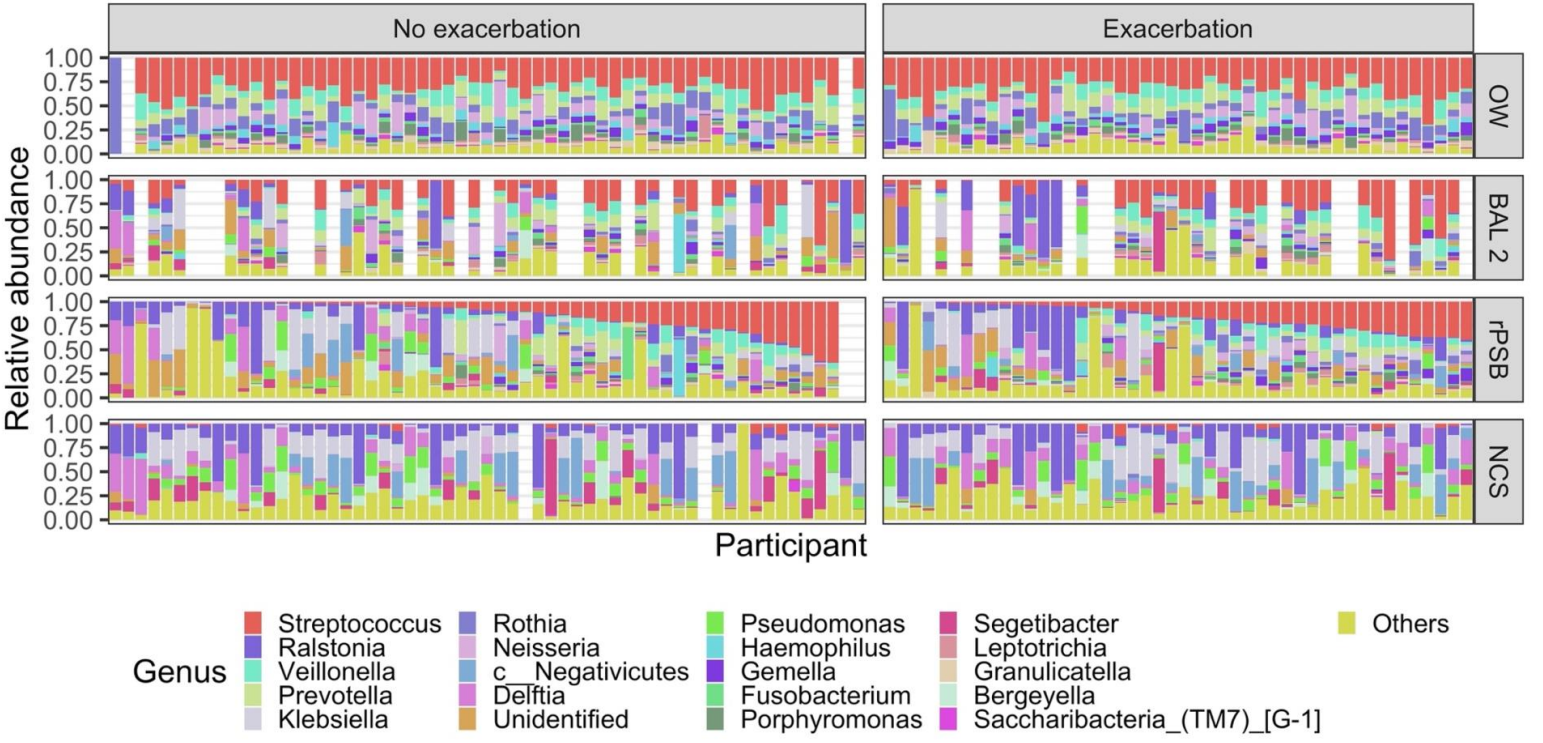


**Figure 7. Bacterial taxonomy at the genus level in participants with COPD with and without exacerbation during follow-up.** Unfiltered data. Taxonomic groups in the legend are sorted in decreasing order based on the average relative frequency of that group in all samples. Each bar represents one participant, ordered in the same position horizontally for all four sample types according to relative abundance of *Streptococcus* in rPSB samples. OW: oral wash; BAL2: second fraction of bronchoalveolar lavage; rPSB: right protected specimen brushes; NCS: negative control samples.

## Diversity

In paper III, there was no difference in alpha or beta diversity between those with and without COPD exacerbations. Other studies have reported inconsistent diversity results when comparing groups with frequent and infrequent exacerbations. Before the publication of paper III, Pragman et al. reported lower alpha diversity (Shannon and Simpson) in frequent exacerbators (36), Millares et al. reported groups to be separated by beta diversity (Bray-Curtis) (35), whereas Ren et al. did not mention differences in diversity (88). Yang et al. reported no separation of groups based on unweighted UniFrac, but found alpha diversity (measured by Chao-1 and number of OTUs) to be lower in high-risk exacerbators. Alpha diversity measured by Shannon index was not lower (262). Bouquet et al. oppositely reported Shannon index (but not number of OTUs) to be lower in those with frequent exacerbations, and a clear separation of groups by weighted and unweighted UniFrac (261). Like for taxonomy and differential abundance, these conflicting results could underpin the negative findings of paper III. They also highlight a need for clinically relevant and meaningful differences for diversity analysis.

A new intriguing suggestion is that microbiota variability (variation in an individual's microbiota over time) in stable disease state, measured by diversity indices, is associated with higher exacerbation frequency and frequent viral infections (261). Due to our cross-sectional design of paper III, we were not able to assess this.

## If not bacteria, what about fungi?

The term microbiota is in this thesis usually limited to investigations into bacterial composition. Other organisms, such as viruses or fungi, could be of importance. In MicroCOPD, we also performed sequencing of the internal transcribed spacer 1 (ITS1), a marker gene for fungi. Some of the results from our *mycobiota* analyses have been published (264). Airway mycobiota is less studied than that of bacterial airway microbiota. A potential association between the mycobiota of stable COPD lungs and

exacerbation frequency was first suggested in 2020: Tiew et al. conducted a longitudinal multicentre study on sputum mycobiota in 337 participants with COPD (265). They reported that very frequent COPD exacerbators (3 or more exacerbations per year in follow-up, n=92) had airway mycobiota profiles discriminated by *Wickerhamomyces*. They also reported that very frequent exacerbators had contrasting beta-diversity compared with nonfrequent exacerbators, although there was no visual clustering of samples based on exacerbator status in ordination space. The differences in mycobiota profiles between the groups remained significant following PERMANOVA testing adjusted for age, sex, smoking pack-year history, body mass index, geographic origin and inhaled corticosteroid use. Total fungal burden, assessed semi-quantitatively by PCR, were comparable between the groups.

In light of these recent findings, it was compelling, when working on this thesis, to briefly check if mycobiota profiles differed between frequent and infrequent exacerbators in the MicroCOPD study as well. Lung mycobiota could be compared in BAL samples from 36 participants with, and 27 participations without, exacerbations in the follow-up. Seven genera (*Mucor*, *Symmetrospora*, *Elmerina*, *Filobasidium*, *Cryptococcus*, *Auricularia*, *Uwebraunia*) were found only in participants who exacerbated, but each of these taxons occurred in only one or two (*Cryptococcus*) individuals in total. By looking at taxonomic distribution (genus level depicted in Figure 8), there was no clear differences between the study groups. As for bacteria, differences between individuals within the groups were more apparent. In the paper by Tiew et al., individual differences were masked by stacked bar-plots. Tiew et al.'s finding of *Wickerhamomyces* in frequent exacerbators was based on Linear discriminant analysis effect size (LEfSe) (266), a tool for differential abundance testing that does not account for compositionality, nor incorporates correction for multiple testing. Of note, *Wickerhamomyces* was not identified in *any* sample in the MicrCOPD study. For analyses of diversity, a rarefaction depth of 1000 was used, leaving only 34 samples for analyses. Bray-Curtis and Jaccard (another non-phylogenetic beta diversity metric) did not support a separation of the two groups (Figure 9). Alpha diversity was *not* found to be different in frequent and non-frequent exacerbators by Tiew et al.. In MicroCOPD, higher evenness (Pielou's evenness, a

non-phylogenetic alpha diversity metric) was found in samples from participants with exacerbations in the follow-up (Figure 10), a tendency that appeared to be dependent on number of exacerbations in the follow-up (Figure 11). Shannon Index appeared to be higher in those with frequent exacerbations (Figure 12), and potentially increasing with number of exacerbations, but the association was not statistically significant (Figure 13)

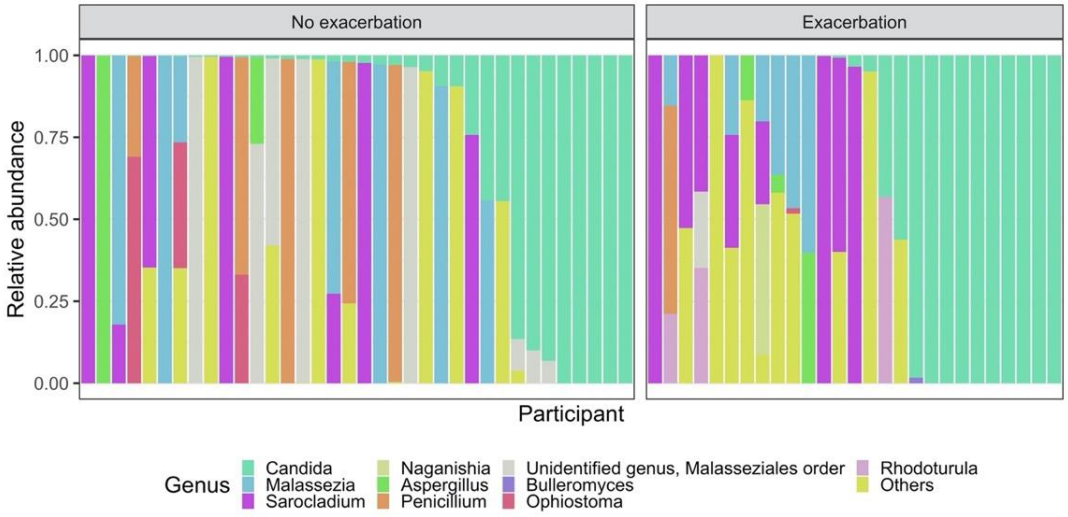


**Figure 8: Fungal taxonomy at the genus level in BAL samples from participants with COPD with and without exacerbation during follow-up**. Taxonomic groups in the legend are sorted in decreasing order based on the average relative frequency of that group in all samples. Each bar represents one participant, ordered according to relative abundance of *Candida* in samples.

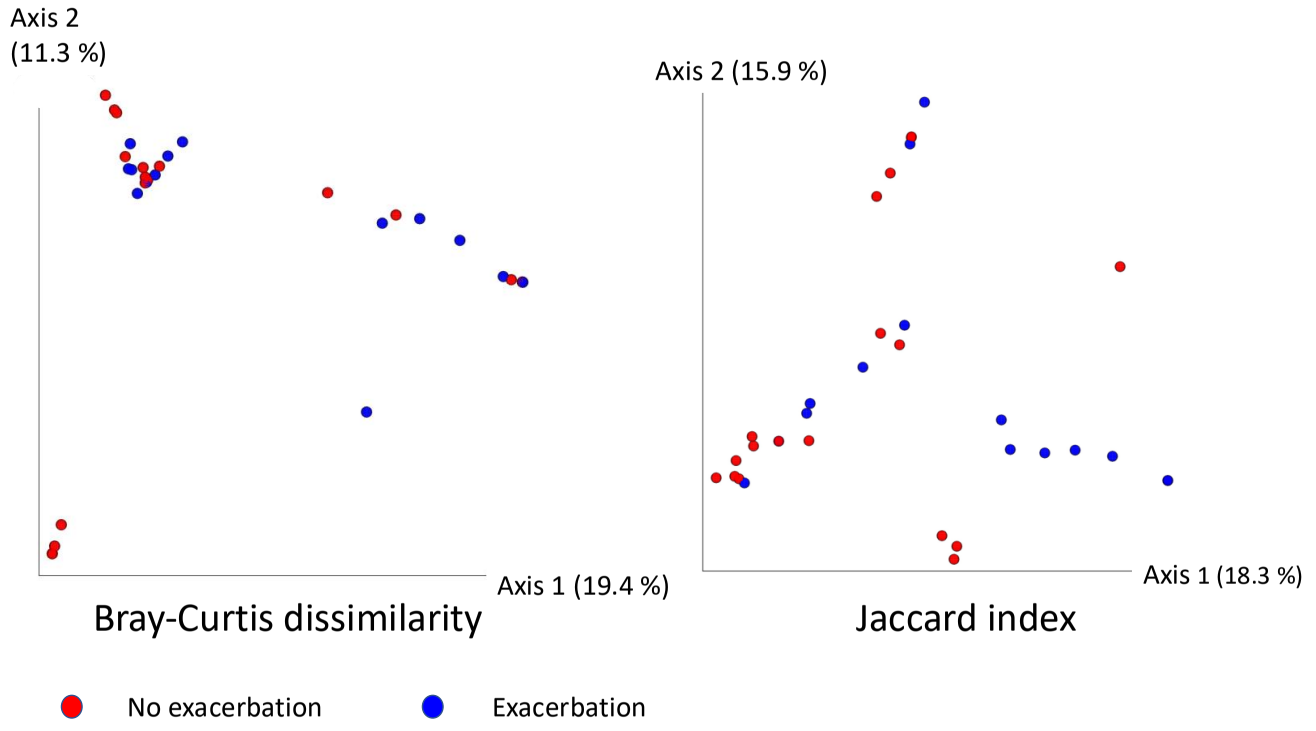


**Figure 9:** PCoA plots of beta diversity, measured by Bray-Curtis dissimilarity and Jaccard index in BAL samples. Each sample is coloured according to exacerbation status (zero versus one or more) during follow-up.

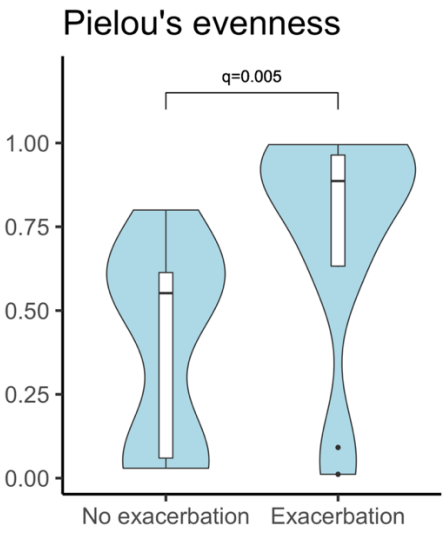


**Figure 10: Alpha diversity measured by Pielou's evenness in BAL samples from 13 participants with and 18 without exacerbation in the follow-up**. 3 samples were omitted due to less than two ASVs. Distribution of samples is illustrated using a combination of violin plots and box plots. Statistical comparison between groups was performed with the Kruskal-Wallis test. q is the p-value corrected for multiple testing (Benjamini-Hochberg).

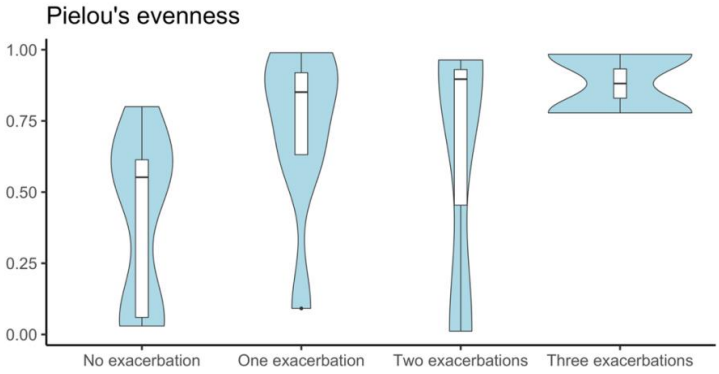


**Figure 11: Alpha diversity measured by Pielou's evenness in BAL samples from 18 participants without exacerbation in the follow-up.** 7 participants with one exacerbation, 3 participants with two exacerbations and 2 participants with 3 exacerbations. 3 samples were omitted due to having less than two observed ASVs. One participant with 6 exacerbations is omitted from the plot. Distribution of samples is illustrated using a combination of violin plots and box plots.

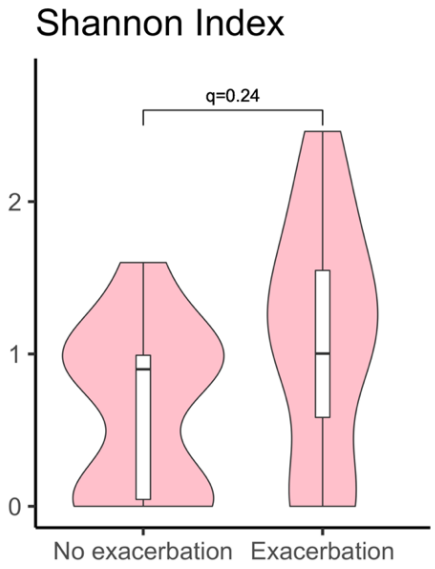


**Figure 12: Alpha diversity measured by Shannon Index in BAL samples from 15 participants with and 19 without exacerbation in the follow-up.** Distribution of samples is illustrated using a combination of violin plots and box plots. Statistical comparison between groups was performed with the Kruskal-Wallis test. q is the p-value corrected for multiple testing (Benjamini-Hochberg).

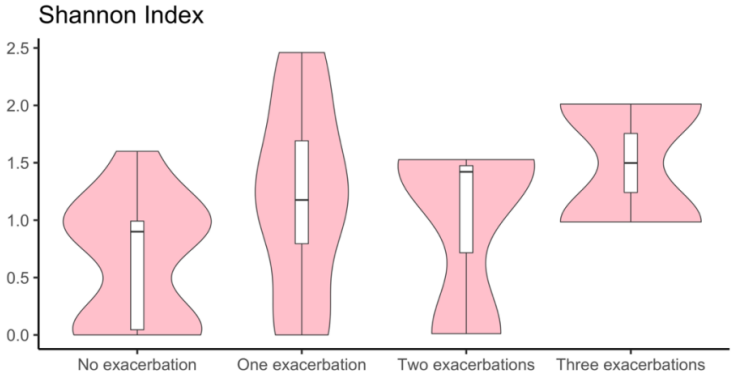


**Figure 13: Alpha diversity measured by Shannon Index in BAL samples from 19 participants without exacerbation in the follow-up**. 8 participants with one exacerbation, 3 participants with two exacerbations and 2 participants with 3 exacerbations. One participant with 4 exacerbations and one with 6 exacerbations were omitted from the plot. Distribution of samples is illustrated using a combination of violin plots and box plots.

This preliminary fungal analysis of samples from the lower airways, show results conflicting to the study of Tiew et al. (265). The mycobiota data presented in this thesis are based on few participants compared to that of the multicentre study, and we could not address fungal burden. Methodological issues specific to DNA extraction, sequencing, and bioinformatic analysis in studies of mycobiota are many (267), and could contribute to differences in findings. However, the study by Tiew et al. has limitations in methodology which are also common in studies of bacterial microbiota. These includes relying on sputum samples (and not differentiating between induced and spontaneous sputum, not including oral samples for comparison) and the use of LEfSe for differential abundance analysis.

In summary, airway microbiota (and mycobiota) analysis in stable COPD did not show an association to exacerbation frequency. Other studies have shown positive, but conflicting findings. Different results can be explained by differences in methods, but could also be due to geographical differences in study populations. To enable comparison between studies, microbiome studies should be more standardised. In the case of the frequent exacerbator phenotype, divergent results support the negative findings in paper III.

# Conclusions

1. Bronchoscopy is a safe procedure with a low risk of severe complications such as mortality, pneumothorax, and bleeding that necessitate intervention. For other complications, we could not find meaningful complication rates, as rates varied substantially between the studies included. Discomfort was difficult to compare between studies. We were not able to conclude on predictors. (Paper I)

2. 25.9% of participants experienced a complication leading to intervention during or immediately following bronchoscopy. Only 1.3% of subjects had a potentially serious complication, all of whom had no sequela, indicating that bronchoscopy is a safe procedure in studies of patients with obstructive lung disease. Sore throat, fever and flu-like symptoms each occurred in roughly half of all subjects. Non-sedation and higher age were significantly associated with more unplanned interventions during bronchoscopy. In participants undergoing more than one bronchoscopy, complications and fever in the first bronchoscopy was often predictive for complications and fever in the second. (Paper II)

3. Individual differences in the lower airway microbiota in persons with COPD far outweigh group differences between frequent and nonfrequent COPD exacerbators. Neither diversity metrices nor analyses of taxonomy in stable state COPD could identify any predictors for frequent exacerbations. (Paper III)

# Implications and future perspectives

The literature review included in this thesis pointed to the need for a well-powered, prospective study with clear definitions of complications and discomfort. The safety study in MicroCOPD was a step in the right direction, but has its limitations as discussed in this thesis. For the topic of safety in COPD research bronchoscopy, there were no papers that could be included at the time of review. The MicroCOPD study implies that research bronchoscopy in COPD is safe, in accordance with the overall conclusion of the SPIROMICS study (259). Although paper II can mainly answer questions related to low-risk research bronchoscopy, the MicroCOPD study procedure is similar to many diagnostic procedures performed at Haukeland University Hospital, and study personnel are also involved in clinical work. Partially the study served as a local quality assessment as well as an evaluation of the research procedure. The specific result that around half of the participants experienced fever sensation following bronchoscopy was surprising. As a direct result of the study, patients undergoing bronchoscopy in the clinic now receive more information that emphasises this side effect. In a way, the findings might be more transferrable to local practice than to the research community as a whole, given the likely impact of the bronchoscopist and local sedation practices. The impact of the MicroCOPD study with regard to patient information is potentially greatest locally. An investigation into specific aspects of diagnostic and therapeutic bronchoscopy at Haukeland University hospital could be interesting, building on the experience from the work in this thesis.

The conclusions of paper III might not be a surprise to a reader who knows the field and its weaknesses, hence the novelty might be questioned. However, all other studies on the microbiome and exacerbations on COPD have reported positive findings. We introduced several improvements to the methodology and presented negative results. This could imply that the microbiota of stable COPD is not associated with frequency of exacerbations, or that the current available and used methods are insufficient for revealing this potentially complex relationship. Conducting additional studies on the topic will be of minimal use unless the remaining methodological issues are resolved.

For future studies I would especially highlight the need for quantitative measures, protected sampling, proper handling of contamination, longitudinal study designs, integration of fungal and viral data, functional data and assessment of microbial viability, and adequate statistical testing. The research community should seek to agree upon standards for data collection and sample processing that enable comparison between studies. That being said, the MicroCOPD study constitutes an important contribution to the respiratory microbiome field with its rigorous focus on methodology. For research into the specific relationship of bacterial microbiota and exacerbation frequency, the MicroCOPD study has reached its potential. A number of MicroCOPD papers exploring other methodological and clinical aspects have been published (95, 134, 136, 184, 264). MicroCOPD has provided a considerable amount of data which is subject to ongoing and future investigations.

The negative findings of paper III might seem disappointing both from a researcher's and participant's perspective. Identifying an association between stable state microbiota and exacerbation frequency could have been a step towards predicting, preventing and improving treatment of exacerbations. In hindsight, knowing that we did not come closer to this long-term goal, one could ask: Was a negative result worth the risk and discomfort of research bronchoscopy? In the MicroCOPD study, bronchoscopy was deemed safe, meaning that the benefit of falsifying previous reports probably outweighs the risks for the individuals. However, if just a single a participant had experienced a severe complication with sequelae, the answer might be opposite. Future airway microbiota studies should absolutely make use of the advantages that bronchoscopy offers, but make sure to assess and secure the safety of research participants.

# Errata

## Paper I:

1. The highest rate bleeding rate was supposed to be 100 %, not 89.9%, as stated in the paper. The rate of 89.9% refers to the report of minimal bleeding in the cited reference, but this reported bleeding is in addition to 8.1% mild and 2.1% moderate bleeding, which sums up to 100%.

2. The section on health care utilisation states “Nine prospective studies (57–59, 61) (65, 68) (70, 84) (86) reported complications that had to be handled by increased health care utilisation”, and should have been “Ten prospective studies (57-61, 65, 68, 70, 84, 86) reported complications that had to be handled by increased health care utilisation”. The missing citation is however included in the same section: “The incidence of health care contacts ranged from 0 to 31%, (59, 60)”. Reference numbers corresponding to those of paper I.

3. The missing reference mentioned above is also missing from Table 4 (supplementary table). Table 4 has been updated with a marked insertion (Table 4_corrected) in Appendix 3 of this thesis.

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# Appendices and papers

Appendix 1: Questionnaires and data collection forms at day of bronchoscopy in English translation.

Appendix 2: Questionnaire for exacerbation follow-up.

Appendix 3: Paper I Supplementary Table 4 with correction.

Paper I

Paper II

Paper III

Appendix 1

*ID number, MicroCOPD:* _____

## Recruitment form and personal information. MicroCOPD.

| | | | | | |
|---|---|---|---|---|---|
| First name, last name | *1.1* | | | Sex | *1.1a* |
| Norwegian national identity number | *1.2a, 1.2b* | | | | |
| ID number BergenCOPD | *1.3* | GeneCOPD ID | *1.3a* | ID, MicroCOPD | *1.4* |
| Recruitment source | *1.5* | | | | |
| Address, postcode and place | *1.6a, 1.6b, 1.6c* | | | | |
| Telephone number | *1.7a, 1.7b, 1.7c, 1.7d* | | | | |

## Re-bronchoscopy (tick):

*1.8*

## Participant group (tick):

| COPD | Control, never-smoke | Control, smoke | Asthma | Other |
|---|---|---|---|---|
| | | | | *1.9* |

## Critical information regarding recruitment

| | |
|---|---|
| Informed about fast | *1.10* |
| Informed about non-ability to drive if sedation is given | *1.11* |
| Questioned about anticoagulants, dual antiplatelet therapy | *1.12* |
| Questioned about artificial heart valve | *1.13* |
| Questioned about antibiotic usage, steroids and exacerbation | *1.14* |

**Bronchoscopic sampling completed?**

| Sample | Tick if completed | Note what is missing, and reason |
|---|---|---|
| Brushes*2 | *1.32* | *1.28* |
| SVL | *1.33* | *1.29* |
| BAL | *1.34* | *1.30* |
| Biopsies | *1.35* | *1.31* |

**Plotted and controlled, date and signature:** _____ *1.27a, 1.27b*

*ID number, MicroCOPD:* _____

## Contraindications.

| | Yes | No |
|---|---|---|
| **BLEEDING RISK** | | |
| Known haemophilia | | *2.1* |
| Blood samples | | *2.2* |
| tpc < $75*10^9$ | | *2.3* |
| INR > 2,0 | | *2.4* |
| Anticoagulants (Marevan, Pradaxa, Xarelto, Eliquis) | | *2.5* |
| Dual antiplatelet therapy, or clopidogrel/plavix/ticagrelor/brilique last 5 days. | | *2.6* |
| Low molecular weight heparin last 24 hours. | | *2.7* |
| **OTHER** | | |
| Artificial heart valve | | *2.8* |
| Myocardial infarction, acute coronary syndrome/unstable angina last 6 weeks | | *2.9* |
| Known severe pulmonary hypertension | | *2.10* |
| SpO2 below 90%, with supplemental oxygen | | *2.11* |
| **STABLE COPD?** | | |
| Hospitalised for COPD last 2 weeks | | *2.12* |
| Antibiotic usage last 2 weeks | | *2.13* |
| Oral glucocorticoids last 2 weeks | | *2.14* |
| Ongoing exacerbation – 2 major or 1 major + 1 minor, 2 subsequent days (relative) | | |
| MAJ: Increased dyspnoea | | *2.15* |
| MAJ: Increased sputum | | *2.16* |
| MAJ: Colour change sputum | | *2.17* |
| MIN: Stuffed/runny nose | | *2.18* |
| MIN: Increased cough or sore throat | | *2.19* |
| MIN: Asthenia | | *2.20* |
| MIN: Increased wheezing sounds from the chest | | *2.21* |
| **THE PATIENT INTENDS TO DRIVE HOME AFTER THE PROCEDURE** | | *2.22* |
| **FAST NOT COMPLETED** | | *2.23* |

In the case of any "yes", the project physician is contacted for individual consideration. If the procedure is conducted despite "yes", the reason is documented here:

*2.24*

_____

*ID number, MicroCOPD:* _____

## Medication and vaccination:

Note all drugs the patient uses, both as-needed and regular medication. No medication: *3.0.*

| Medication name | Administration form | Dose (unit) | Dosage (*number of doses per 24 hours, or B for as-needed*) | Last dosage given (time in last 24h) | Start date if cure |
|---|---|---|---|---|---|
| *3.1a* | *3.1b* | *3.1c, 3.1d* | *3.1e* | *3.1f* | *3.1g* |
| *3.2a* | *3.2b* | *3.2c, 3.2d* | *3.2e* | *3.2f* | *3.2g* |
| *3.3a* | *3.3b* | *3.3c, 3.3d* | *3.3e* | *3.3f* | *3.3g* |
| *3.4a* | *3.4b* | *3.4c, 3.4d* | *3.4e* | *3.4f* | *3.4g* |
| *3.5a* | *3.5b* | *3.5c, 3.5d* | *3.5e* | *3.5f* | *3.5g* |
| *3.6a* | *3.6b* | *3.6c, 3.6d* | *3.6e* | *3.6f* | *3.6g* |
| *3.7a* | *3.7b* | *3.7c, 3.7d* | *3.7e* | *3.7f* | *3.7g* |
| *3.8a* | *3.8b* | *3.8c, 3.8d* | *3.8e* | *3.8f* | *3.8g* |
| *3.9a* | *3.9b* | *3.9c, 3.9d* | *3.9e* | *3.9f* | *3.9g* |
| *3.10a* | *3.10b* | *3.10c, 3.10d* | *3.10e* | *3.10f* | *3.10g* |
| *3.11a* | *3.11b* | *3.11c, 3.11d* | *3.11e* | *3.11f* | *3.11g* |

| | **Approximate month/year** |
|---|---|
| **When did you last receive a flu shot?** | *3.12* |
| **When did you last receive antibiotic treatment?** | *3.13* |
| **When was your latest cortisone/prednisolone cure?** | *3.14* |

*ID number, MicroCOPD:* _____

## Conditions/diseases (active treatment, current symptoms, sequelae etc)

| Disease Yes | No | Diagnosis | When diagnosed (years since) |
|---|---|---|---|
| | 4.1 | Chronic obstructive pulmonary disease (COPD) | 4.1a |
| | 4.2 | Emphysema | 4.2a |
| | 4.3 | Chronic bronchitis | 4.3a |
| | 4.4 | Asthma | 4.4a |
| | 4.5 | Lung fibrosis | 4.5a |
| | 4.6 | Cystic fibrosis | 4.6a |
| | 4.7 | Sarcoidosis | 4.7a |
| | 4.8 | Lung cancer | 4.8a |
| | 4.9 | Tuberculosis | 4.9a |

*Conditions in airways are to be verified through medical history taking/spirometry/medical records and are regarded as diagnoses given/verified at the time of examination.*

| *CONDITION* | Yes | No | *CONDITION* | Yes | No |
|---|---|---|---|---|---|
| Diabetes mellitus | | 4.10 | Depression with regular use of medication | | 4.33 |
| Myocardial infarction | | 4.11 | Other psychiatric illness | | 4.34 |
| Angina | | 4.12 | which? | | 4.35 |
| Intermittent claudication | | 4.13 | Muscle disease with regular use of medication | | 4.36 |
| Heart valve condition | | 4.14 | which? | | 4.37 |
| Heart failure | | 4.15 | **Active known cancer (diagnosed/treated last 5 years)** | | |
| Cerebral infarction or bleeding | | 4.16 | Lung cancer | | 4.38 |
| Other known neurological disease | | 4.17 | GI cancer | | 4.39 |
| which? | | 4.18 | Breast cancer | | 4.40 |
| Gastric ulcer | | 4.19 | Endometrial cancer (NOT dysplasia only) | | 4.41 |
| Hepatic disease | | 4.20 | Cancer in gonads (testes/ovaries) | | 4.42 |
| which? | | 4.21 | Prostate cancer | | 4.43 |
| Kidney disease | | 4.22 | Blood cancer, leukaemia | | 4.44 |
| which? | | 4.23 | Lymphoma | | 4.45 |
| High blood pressure, treated hypertension | | 4.24 | Skin cancer (not including treated basalioma) | | 4.46 |
| | | | Other type of cancer | | 4.47 |
| **Inflammatory diseases in need of therapy** | | | which | | 4.49 |
| Rheumatoid arthritis | | 4.26 | **Other diseases (active treatment, physician-given diagnose) – write here:** | | |
| Psoriasis arthritis | | 4.27 | | | 4.50 |
| Systemic lupus erythematosus | | 4.28 | | | 4.51 |
| Polymyalgia rheumatica | | 4.29 | | | 4.52 |
| Ulcerous colitis/Mb Crohn | | 4.30 | | | 4.53 |
| Disease in skeleton or joints with regular use of medication, including osteoporosis | | 4.31 | | | 4.54 |
| Which? | | 4.32 | | | 4.55 |

*Comorbidities should, to the greatest extent possible, be verified through medical history taking or medical record review.*

*ID number, MicroCOPD:* _____

## Marital status, children, education, menopause, domestic animals

| | | | |
|---|---|---|---|
| 1. Are you (one tick): | | Married/Registered partner | 5.1a |
| | | Widow/widower | 5.1b |
| | | Cohabitant | 5.1c |
| | | Divorced, live alone | 5.1d |
| | | Unmarried/single | 5.1e |
| 2. If you have children, how many? | | *number* | 5.2 |
| 3. Which education level best suits you? | | Compulsory education | 5.3a |
| | | High school/vocational training | 5.3b |
| | | 3 years of higher education/university | 5.3c |
| | | ≥4 years of higher education/university | 5.3d |
| 4. For women, do you still experience regular periods? | Yes | No | 5.4 |
| 4. a. If no, when did you reach menopause? | *years ago* | | 5.5 |
| 5. Do you keep domestic animals or birds? | Yes | No | 5.6 |
| 5. b. Which domestic animal(s)/bird(s) do you have? | | | 5.6a1 – 5.6a4 |
| 5. c. Have you kept domestic animals/birds at home before? | Yes | No | 5.6b |
| 5. d Which domestic animal(s)/bird(s) did you have before? | | | 5.6c1 - 5.6c4 |

## Arterial blood gas and pulse oximetry

| | Yes | No | |
|---|---|---|---|
| Does the patient receive continuous oxygen | | | 5.7 |
| Oxygen supplied in the 30 minutes prior to puncture (litres/min) | | | 5.8 |
| **Blood gas results** | | | |
| $FiO_2$, oxygen fraction, room air = 0,21 | | | 5.9 |
| pH | | | 5.10 |
| Oxygen tension ($PaO_2$, kPa) | | | 5.11 |
| Carbon acid tension ($PaCO_2$, kPa) | | | 5.12 |
| arterial saturation (%) | | | 5.13 |
| bicarbonate, mmol/l | | | 5.14 |
| carbon monoxide, % | | | 5.15 |
| haemoglobin, g/dl | | | 5.16 |
| Blood gas not performed because (perform pulse oximetry, note in bronchoscopy form) | | | |
| Refuses | | | 5.17 |
| > 6 attempts | | | 5.18 |
| Apparatus failure | | | 5.19 |

*ID number, MicroCOPD:* _____

# Lung function testing, height, weight

1. Chosen spirometer: _____ *6.1*

2. Technician (four-character code): ☐☐☐☐ *6.2*

3. Weight of participant (kg): ☐☐☐.☐ *6.3*

4. Height of participant (in whole cm) ☐☐☐ *6.4*

5. Given Ventolin? Yes No *6.5*

5.1 Time: ☐☐:☐☐ *6.6*

6. Time test start: ☐☐:☐☐ *6.7*

7.a Best FEV1 _____ (litres) *6.8* 7b. Best $FEV_1$ % of predicted_____ *6.9*

8.a Best FVC _____ (litres) *6.10* 8b. Best FVC % of predicted_____ *6.11*

9. Which reference values were applied? _____ *6.12*

*ID number, MicroCOPD:* _____

# COPD exacerbations, smoking habits, alcohol

**1.** Number of exacerbations in the last 12 months requiring antibiotics/steroids or hospital admission?

_____ (number of exacerbations) *7.1*

*If yes, answer question 2, if not, move on to question 3.*

**2.** If one or more therapy requiring exacerbations in the last 12 months – how many of them required acute hospitalisation?

_____ (number of admissions) *7.2*

**3a** Do you smoke daily now? Yes No
**If yes, answer 3b, if no move to 3d** *7.3*

**3b** Do you smoke cigarettes daily? (roll-your-own or manufactured)? Yes No
**If yes, move to 3f, if no move to 3c** *7.4*

**3c** What do you use to smoke tobacco? pipe
cigar
**Move to 3f** *7.5*

**3d** Have you smoked daily before? Yes No
**If yes move to 3e, if no move to 3i** *7.6*

**3e** How long since you quit?
Less than three months
Between three months and one year
One to five years
More than five years *7.7*

**3f** How many years have you smoked daily? ⌊_⌋⌊_⌋ *7.8* Number of years

**3g** How many cigarettes do you smoke or did you smoke daily? (give the number per day, both roll-your-own and manufactured) **Move to question 4.** ⌊_⌋⌊_⌋ *7.9* Number of units

**3i** Do you smoke cigarettes *once in a while*, or have you smoked cigarettes once in a while before? **If yes, move to 3j and 3k, if not move to question 4.** Yes Before No *7.13*

**3j** For how long have you smoked *once in a while*? ⌊_⌋⌊_⌋ *7.14* Number of years

**3k** How many cigarettes do/did you smoke in the course of a regular week? ⌊_⌋⌊_⌋ *7.15* Number of units

**4.** Number of cigarettes or tobacco units (not snus) in the last 24 hours _____ number of units. *7.10*

**5.** Number of hours since you smoked or used tobacco _____ (number of hours) *7.11*

**6.** How many units of alcohol do you consume in the course of an average week?

_____ (number of alcohol units) *7.12*

*ID number, MicroCOPD:* _____

## CAT COPD assessment test (copyright GSK):

For each item below, place a mark (X) in the box that best describes your current situation. Please ensure that you only select one response for each question

Example:

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| I am very happy | 0 | 1 | 2 | 3 | 4 | 5 | I am very sad |

| | | | | | | | | SCORE |
|---|---|---|---|---|---|---|---|---|
| I never cough | 0 | 1 | 2 | 3 | 4 | 5 | I cough all the time | 8.1 |
| I have no phlegm (mucus) in my chest at all | 0 | 1 | 2 | 3 | 4 | 5 | My chest is full of phlegm (mucus) | 8.2 |
| My chest does not feel tight at all | 0 | 1 | 2 | 3 | 4 | 5 | My chest feels very tight | 8.3 |
| When I walk up a hill or a flight of stairs, I am not out of breath | 0 | 1 | 2 | 3 | 4 | 5 | When I walk up a hill or a flight of stairs, I am completely out of breath | 8.4 |
| I am not limited to doing any activities at home | 0 | 1 | 2 | 3 | 4 | 5 | I am completely limited to doing all activities at home | 8.5 |
| I am confident leaving my home despite my lung condition | 0 | 1 | 2 | 3 | 4 | 5 | I am not confident leaving my home at all because of my lung condition | 8.6 |
| I sleep soundly | 0 | 1 | 2 | 3 | 4 | 5 | I do not sleep soundly because of my lung condition | 8.7 |
| I have lots of energy | 0 | 1 | 2 | 3 | 4 | 5 | I have no energy at all | 8.8 |
| | | | | | | | TOTAL SCORE | 8.9 |

## Motivation for participation

Why did you wish to take part in this project (open question, answer in free-form text)?

8.10

---

## Expectations

On a scale from zero to 10, where 10 is the worst you can imagine, and 0 is nothing. How much do you dread this examination? (whole numbers, **no comment**)

8.11

---

*ID number, MicroCOPD:* _____

## Assessment of chronic dyspnoea (mMRC scale)

**First**
Are you restricted from walking due to other condition than breathlessness?

Yes ☐ No ☐ *9.0*

*If yes, move pass the next question, if no:*

**Give the one answer that is correct for you (mark only *one*)** 9.1

☐ I am too breathless to leave the house or I am breathless when dressing.

☐ I stop for breath after walking about 100 metres or after a few minutes on level ground

☐ On level ground, I walk slower than people of the same age because of breathlessness, or have to stop for breath when walking at my own pace.

☐ I get short of breath when hurrying on level ground or walking up a slight hill

☐ I only get breathless with strenuous exercise

## Assessment of dyspnoea before bronchoscopy (Borg scale) *9.2*

| How severe is your breathlessness? | | | Tick |
|---|---|---|---|
| **0** | Nothing at all | "No intensity" | |
| **0,3** | | | |
| **0,5** | Very, very slight | Just noticeable | |
| **0,7** | | | |
| **1** | Very slight | | |
| **1,5** | | | |
| **2** | Slight | Light | |
| **2,5** | | | |
| **3** | Moderate | | |
| **4** | | | |
| **5** | Severe | Heavy | |
| **6** | | | |
| **7** | Very severe | | |
| **8** | | | |
| **9** | | | |
| **10** | Extremely severe | "Strongest intensity" | |
| **11** | | | |
| * | Absolute maximum | Highest possible | |

Borg CR10 scale. 

*ID number, MicroCOPD:* _____

# Bronchoscopy date: ____/____/20___ 10.0

| | | | | | |
|---|---|---|---|---|---|
| **Safety** | | | | | |
| Contraindications checked | 10.1 | Allergies, asked* | 10.2 | Blood samples date (safety) | 10.2a |
| Tpc *$10^9$ | 10.3 | Hb, mg/l | 10.4 | INR | 10.5 |
| SpO2 WITHOUT supplemental oxygen (before start, %) | 10.7b | SpO2 WITH supplemental oxygen (before start, %) | 10.6 | O2 supplied (l/min | 10.7 |
| BP before anaesthesia | 10.8 | BP after anaesthesia | 10.9 | | |
| **Operators, equipment** | | | | | |
| Operator 1, four-character code | 10.10 | Nurse 1, four-character code | 10.11 | | |
| Bronchoscope 1 | 10.12 | Rack | 10.13 | | |
| **Drugs** | | | | | |
| Lidocaine 10 mg/spray, number of applications | 10.14 | | | | |
| Bronchodilation prior to procedure | *Drug(s)* | *Amount + unit* | *Indication* | | |
| *Fill in here if given:* | 10.15 | 10.16, 10.16a | 10.17 | | |
| Alfentanil preop., mg | 10.18 | Supplemental alfentanil perop, mg | 10.19 | | |
| Midazolam preop, mg | 10.20 | Supplemental midazolam perop, mg | 10.21 | | |
| Applied lidocaine during procedure, in millilitre (20 mg/ml) | 10.22 | Applied adrenaline, 0,1 mg/ml, place, amount | 10.23 | | |
| **Procedure start and end** | | | | | |
| Time, start (passing of **vocal cords**) | 10.24 | Time end (scope withdrawn) | 10.25 | | |

*ID number, MicroCOPD:* _____

| **Sampling** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Negative controls of fluid?** | | 11.1 | **Inspection** completed** | 11.2 | **Normal** (not norm. next page) | | 11.3 |
| **Gingival samples taken.** | | 11.3a | Comment gingiva | | | 11.3b | |
| **Oral wash, type & fluid amount** | | | 11.4a, 11.4b, | | **Oral wash return (ml)** | | 11.5 |
| **Brushes** | **Order** | **Lobe** | | | **Segment** | | **Number of brushes** |
| Right | 11.6a | | 11.6 | | 11.7 | | 11.8 |
| Left | 11.9a | | 11.9 | | 11.10 | | 11.11 |

| **Lavage** | **Order** | **Lobe** | **Segm.** | **Type of fluid** | **BAL or SVL?** | **Installed (ml)** | **Return (ml)** |
|---|---|---|---|---|---|---|---|
| Right | 11.18a | 11.18 | 11.19 | 11.20 | 11.21 | 11.22 | 11.23 |
| Left | 11.12a | 11.12 | 11.13 | 11.14 | 11.15 | 11.16 | 11.17 |

| **Endobrochial biopsy** | **Place (lobe, carina level and segment)** | **Type of forceps** | **Sent to (GMA, freezer, mitoc., other)** |
|---|---|---|---|
| number 1 | 11.24 | 11a | 11a1 |
| number 2 | 11.27 | 11b | 11b1 |
| number 3 | 11.30 | 11c | 11c1 |
| number 4 | 11.33 | 11d | 11d1 |
| number 5 | 11.36 | 11e | 11e1 |
| number 6 | 11.39 | 11f | 11f1 |
| number 7 | 11.41a | 11g | 11g1 |
| number 8 | 11.41c | 11h | 11h1 |

| **Termination** | | |
|---|---|---|
| Complicated | 11.42, 11.43 | *Must fill out complication form* |
| When can the patient eat (time) | 11.44 | |
| Observation time completed, time, four-character code | 11.45 | 11.46 |

* anaesthesia, sedation

** vocal cords, carina, inspection of all lobes and segmental ostia

*ID number, MicroCOPD:* _____

# Bronchoscopy event form

Study personnel present: _____ *12.1a - h*

| **Event:** | **Time, duration, assumed cause and intervention** |
|---|---|
| **No event (mark)** *12.2* | |
| **Cough** *12.3, 12.3a* | |
| **Dyspnoea** *12.4, 12.4a* | |
| **Oxygen desaturation >4 %<br>or to <90 %** *12.5, 12.5a* | |
| **Change in BP/Heart rate** *12.6, 12.6a* | |
| **Bleeding** *12.7* | |
| **Other serious complication** *12.8* | |
| **Other events** *12.9* | |

*ID number, MicroCOPD:* _____

## Subject experience of bronchoscopy

| Question | Immediately after | After the observation | After 1 week |
|---|---|---|---|
| A. What do you think of the procedure now? Have you had any discomforts (which?) **(Open question)** | *13.1a* | *13.1b* | *13.1c* |
| B. How **uncomfortable** did you find this experience, taking into consideration everything that has happened until now, on a scale from 0 to 10, where 10 is the most uncomfortable you can imagine, and 0 is no discomfort. | *13.2a* | *13.2b* | *13.2c* |
| C. For how long do you think the procedure lasted? | *13.3a* | | |
| 1. How short of breath are you now? (Borg scale), 0-10 (show Borg scale) | *13.4a* | *13.4b* | *13.4c* |
| 2. If you were asked to participate in a **new** research project involving the **same procedure**, would you participate? (if yes, go to question 4, if no, ask question 3) | *13.5a* | *13.5b* | *13.5c* |
| 3. If your doctor advised you to undergo this type of procedure, would you then have done it **again**? | *13.6a* | *13.6b* | *13.6c* |
| 4. Do you have **a sensation of fever** in your body now, or have you had fever/fever sensation in relation to bronchoscopy or after the procedure? | *13.7a* | *13.7b* | *13.7c* |
| 5. Did you cough **blood** or red/light red saliva? | *13.8a* | *13.8b* | *13.8c* |
| **6. Have you, after the procedure, experienced** | | | |
| a. Increased breathlessness, dyspnoea or tightening of the chest? (*Synonymous words*)? | *13.9a* | *13.9b* | *13.9c* |
| b. Increased sputum? | *13.10a* | *13.10b* | *13.10c* |
| c. Sputum colour change? | *13.11a* | *13.11b* | *13.11c* |
| d. Increased rhinitis/stuffed nose? | *13.12a* | *13.12b* | *13.12c* |
| e. Increased wheezing chest sounds? | *13.13a* | *13.13b* | *13.13c* |
| f. Sore throat/increased cough? | *13.14a* | *13.14b* | *13.14c* |
| g. Increased fatigue/lack of initiative? | *13.15a* | *13.15b* | *13.15c* |
| f. flu symptoms (fever, muscle/joint ache, headache, reduced general condition)? | *13.16a* | *13.16b* | *13.16c* |
| 7. Have you, after the procedure, needed to **seek a doctor/call** (unscheduled), use **antibiotics**, receive cortisone/**prednisolone** or be admitted to **hospital**? (if yes, note what, and cause) | | | *13.17, 13.17a* |
| 8. Have you, in relation to the procedure or after received any new treatment? In case, which? (type of treatment, if drug – dosage etc) | | | *13.18* |
| 9. Note if the participant has been in contact with a physician or the study personnel outside of standard follow-up – reason for contact, date and intervention. | | | *13.19* |

Appendix 2

## Ringeskjema OLS forverrelser for pasienter i studien MikroKOLS

Pasient status: ☐ KOLS ☐ Astma

Dato for første bronkoskopi (dd-MMM-åå): _____

evt Dato for andre bronkoskopi (dd-MMM-åå): _____

Planlagt ca dato for første telefon: (dd-MMM-åå): _____

Planlagt ca dato for andre telefon: (dd-MMM-åå): _____

Planlagt ca dato for tredje telefon: (dd-MMM-åå): _____

Planlagt ca dato for siste telefon: (dd-MMM-åå): _____

***Faste*** *medisiner for obstruktiv lungesykdom (fylles ut av bronkoskopør):*

Navn (x: Seretide diskus) Styrke (x: 50/500ug/d) Dosering (x: 1x2)

_____

_____

_____

_____

_____

Dato for forrige kontakt (dd-MMM-åå): _____

Dato for oppnådd telefonkontakt (dd-MMM-åå):_____

1) INNLEGGELSER

"**Har du siden forrige telefonkontakt vært innlagt på sykehus?**" ☐ Nei ☐ Ja

Hvis ja:

"Hvor mange ganger siden forrige telefonkontakt har du vært innlagt?": _____

*For første innleggelse:*

"Var du innlagt for en forverrelse av din KOLS/astma?" ☐ Nei ☐ Ja

Hvis Nei:

"Hva var årsaken til innleggelsen?":_____

"Husker du omtrent hvilken dato du ble innlagt?" (ca dd-MMM-åå):_____

"Husker du om du fikk antiobiotika (Som Amoxicillin eller lignende) eller prednisolon under eller etter innleggelsen?"

☐ Begge deler ☐ Kun Prednisolon ☐ Kun antiobiotika

*For evt andre innleggelse:*

"Var du innlagt for en forverrelse av din KOLS/astma?" ☐ Nei ☐ Ja

Hvis Nei:

"Hva var årsaken til innleggelsen?":_____

"Husker du omtrent hvilken dato du ble innlagt?" (ca dd-MMM-åå):_____

"Husker du om du fikk antiobiotika (Som Amoxicillin eller lignende) eller prednisolon under eller etter innleggelsen?"

☐ Begge deler ☐ Kun Prednisolon ☐ Kun antiobiotika

*For evt tredje innleggelse:*

"Var du innlagt for en forverrelse av din KOLS/astma?" ☐ Nei ☐ Ja

Hvis Nei:

"Hva var årsaken til innleggelsen?":_____

"Husker du omtrent hvilken dato du ble innlagt?" (ca dd-MMM-åå):_____

"Husker du om du fikk antiobiotika (Som Amoxicillin eller lignende) eller prednisolon under eller etter innleggelsen?"

☐ Begge deler ☐ Kun Prednisolon ☐ Kun antiobiotika

2) BESØK I AKUTTMOTTAK MEN IKKE INNLAGT (som regel definert som under 6 timer i mottak)

"**Har du vært innom sykehusets akuttmottak grunnet forverrelse av din KOLS/astma, men ikke hatt behov for innleggelse?**" ☐ Nei ☐ Ja

Hvis ja: "Hvor mange ganger?": _____

*For første hendelse:*
"Husker du omtrent hvilken dato du var i akuttmottaket?": _____

"Husker du om du fikk antiobiotika (Som Amoxicillin eller lignende) eller prednisolon under eller etter besøket?"
☐ Begge deler ☐ Kun Prednisolon ☐ Kun antiobiotika

*For evt andre hendelse:*
"Husker du omtrent hvilken dato du var i akuttmottaket?": _____

"Husker du om du fikk antiobiotika (Som Amoxicillin eller lignende) eller prednisolon under eller etter besøket?"
☐ Begge deler ☐ Kun Prednisolon ☐ Kun antiobiotika

*For evt tredje hendelse:*
"Husker du omtrent hvilken dato du var i akuttmottaket?": _____

"Husker du om du fikk antiobiotika (Som Amoxicillin eller lignende) eller prednisolon under eller etter besøket?"
☐ Begge deler ☐ Kun Prednisolon ☐ Kun antiobiotika
3) BESØK HOS FASTLEGEN (eller tilsvarende på fastlegekontoret)

"**Har du siden forrige telefonkontakt vært hos fastlegen og fått behandling for en forverrelse av din KOLS/astma?**" ☐ Nei ☐ Ja
Hvis ja:

"Hvor mange ganger siden forrige telefonkontakt har du vært hos fastlegen?":
_____

*For første hendelse:*
"Husker du omtrent hvilken dato du var hos fastlegen?": _____

"Husker du om du fikk antiobiotika (Som Amoxicillin eller lignende) eller prednisolon etter besøket?"
☐ Begge deler ☐ Kun Prednisolon ☐ Kun antiobiotika

*For evt andre hendelse:*
"Husker du omtrent hvilken dato du var hos fastlegen?": _____

"Husker du om du fikk antiobiotika (Som Amoxicillin eller lignende) eller prednisolon etter besøket?"
☐ Begge deler ☐ Kun Prednisolon ☐ Kun antiobiotika

*For evt tredje hendelse:*
"Husker du omtrent hvilken dato du var hos fastlegen?": _____

"Husker du om du fikk antiobiotika (Som Amoxicillin eller lignende) eller prednisolon etter besøket?"
☐ Begge deler ☐ Kun Prednisolon ☐ Kun antiobiotika

"**Har du siden forrige telefonkontakt vært i telefonkontakt med fastlegen og fått behandling for en forverrelse av din KOLS/astma?**" ☐ Nei ☐ Ja

Hvis ja: "Hvor mange ganger?": _____

*For første hendelse:*
"Husker du omtrent hvilken dato du ringte fastlegen?": _____

"Husker du om du fikk antiobiotika (Som Amoxicillin eller lignende) eller prednisolon etter telefonkontakten?"
☐ Begge deler ☐ Kun Prednisolon ☐ Kun antiobiotika

*For evt andre hendelse:*
"Husker du omtrent hvilken dato du ringte fastlegen?": _____

"Husker du om du fikk antiobiotika (Som Amoxicillin eller lignende) eller prednisolon etter telefonkontakten?"
☐ Begge deler ☐ Kun Prednisolon ☐ Kun antiobiotika

*For evt tredje hendelse:*
"Husker du omtrent hvilken dato du ringte fastlegen?": _____

"Husker du om du fikk antiobiotika (Som Amoxicillin eller lignende) eller prednisolon etter telefonkontakten?"
☐ Begge deler ☐ Kun Prednisolon ☐ Kun antiobiotika

4) BESØK PÅ AKUTT LEGEVAKT

"**Har du siden forrige telefonkontakt vært på akutt legevakten og fått behandling for en forverrelse av din KOLS/astma?**" ☐ Nei ☐ Ja
Hvis ja:

"Hvor mange ganger siden forrige telefonkontakt har du vært på legevakten?": _____

*For første hendelse:*
"Husker du omtrent hvilken dato du var på legevakten?": _____

"Husker du om du fikk antiobiotika (Som Amoxicillin eller lignende) eller prednisolon etter besøket?"

☐ Begge deler ☐ Kun Prednisolon ☐ Kun antiobiotika

*For evt andre hendelse:*

"Husker du omtrent hvilken dato du var på legevakten?": _____

"Husker du om du fikk antiobiotika (Som Amoxicillin eller lignende) eller prednisolon etter besøket?"

☐ Begge deler ☐ Kun Prednisolon ☐ Kun antiobiotika

*For evt tredje hendelse:*

"Husker du omtrent hvilken dato du var på legevakten?": _____

"Husker du om du fikk antiobiotika (Som Amoxicillin eller lignende) eller prednisolon etter besøket?"

☐ Begge deler ☐ Kun Prednisolon ☐ Kun antiobiotika

"**1000 takk for hjelpen, vi ringer deg tilbake om ca 3 måneder!**"

Appendix 3

Supplementary Table 4 with marked correction

| *Complication type* | ref # | First author | Journal | Year published | Population | Exclusion/inclusion criteria | Sample size | Mean complication prevalence | Remarks |
|---|---|---|---|---|---|---|---|---|---|
| ***Death*** | | | | | | | | | |
| | 53 | Dunagan | Chest | 1997 | Bone marrow recipients | Excluded: Patients whose clinical assessment indicated that oxygen and haemodynamic status might be compromised by the procedure. | 71 | 2.8% | 24-hour mortality. The patients who died experienced extensive bleeding during the procedure. |
| | 54 | Shannon | Bone Marrow Transplant | 2010 | Bone marrow recipients | Included: Patients whose SaO2 exceeded 90% on room air or supplemental O2. For TBB with BAL: platelet count > 80 x106 /mcgL. | 501 | 0.0% | 24-hour mortality. States no procedure-related deaths. |
| | 55 | D'Ippolito | Monaldi Archives for Chest Disease | 2007 | Elderly patients | Excluded: Platelet count <50 000, APTT >=50 s or PTT =<75%, and haemodynamic instability. | 301 | 0.0% | |
| | 56 | Diaz-Guzman | Respiration | 2009 | Pulmonary hypertension patients | Included: Patients with a diagnosis of PH defined as (1) mean PAP >25 mmHg measured by right heart catheterization or (2) right ventricular systolic pressure >40 mm Hg estimated by Doppler echocardiography and clinical evidence of heart failure. Controls: Patients that did not meet criteria for PH + underwent bronchoscopy by the same physician within 48 hours of the study patients. | 90 | 0.0% | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 57 | Dang | Internal Medicine Journal | 2012 | Consecutive patients referred to bronchoscopy | Excluded: Patients with hypersensitivity or allergies to anaesthetic medications, opioids or benzodiazepines, with brady- or tachycardia (resting heart rate <60 or >100 bpm), or systolic blood pressure of <100 or >180 mmHg), concurrent psychological disorders, those already on high doses of opioids or benzodiazepines, and oxygen requirements of >4 L/min O2 at rest. | 539 | 0.0% | |
| | 58 | Jain | Chest | 2004 | Immunocompromised patients with lung infiltrates | Included: Immunocompromised patients with the presence of either a focal or diffuse pulmonary infiltrate, referred and giving informed consent to bronchoscopy. Excluded: HIV positive patients, lung transplant recipients. | 104 | 0.0% | |
| | 59 | Grendelmeier | Swiss Medical Weekly | 2011 | Elective bronchoscopy patients | Excluded: Patients who were intubated and patients with known propofol allergy/intolerance. | 440 | 0.0% | |
| | 60 | Schlatter | The European respiratory journal | 2011 | Elective bronchoscopy patients | Excluded: Intubated patients, patients receiving interventional bronchoscopy (e.g. laser or stent), patients with known hydrocodone or propofol allergy/intolerance. | 300 | 0.0% | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 61 | Grendelmeier | The European respiratory journal | 2014 | Patients undergoing non-emergency bronchoscopy | Excluded: ICU/emergency bronchoscopy, patients aged <18 years, intubated or isolated patients, patients with known propofol allergy/intolerance, pregnant or breastfeeding patients, and patients with a mental disorder preventing appropriate judgment concerning study participation. | 702 | 0.0% | |
| ***Bleeding*** | | | | | | | | | |
| | 58 | Jain | Chest | 2004 | Immunocompromised patients with lung infiltrates | Included: Immunocompromised patients with the presence of either a focal or diffuse pulmonary infiltrate, referred and giving informed consent to bronchoscopy. Excluded: HIV positive patients, lung transplant recipients. | 104 | 13.5% | |
| | 59 | Grendelmeier | Swiss Medical Weekly | 2011 | Elective bronchoscopy patients | Excluded: Patients who were intubated and patients with known propofol allergy/intolerance. | 440 | 2.5% | Minor bleeding. Not defined. |
| | 62 | Carr | Respiration | 2012 | Patients with low risk of bleeding | Excluded: Patients with active pulmonary bleeding (haemoptysis), personal/family history or physical evidence of bleeding tendencies (petechiae, purpura, ecchymosis, haematoma), ever having anti-platelet therapy (aspirin, clopidogrel) or anti-coagulation therapy (warfarin, heparin), history or physical evidence of liver disease (hepatitis, cirrhosis, hepatomegaly, ascites, jaundice), platelet count <20 *10^3. | 234 | 89.7%-8.1%-2.1% | Minimal - mild - moderate |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 63 | Ni | Chang Gung medical journal | 2010 | Patients undergoing diagnostic bronchoscopy | Excluded: patients aged <18 years, ASA IV or V, neurologic disorders or other conditions contributing to difficulty in assessing a conscious response, FVC < 15 ml/kg body weight, FEV1 < 1000 ml, or FEV1/FVC < 35%, patients with known allergy to study drugs, and patients with glaucoma. | 88 | 26.1% | In need of adrenalin spray. |
| | 64 | Herth | Chest | 2002 | Patients aged > 40 undergoing TBB | Excluded: Patients with warfarin/heparin use last 2 weeks, known bleeding disorder, or TPK<80 000cells/mcL. | 1217 | 4.7% | |
| | 65 | von Bartheld | JAMA | 2013 | Patients with sarcoidosis | Excluded: Patients with obvious organ involvement of sarcoidosis with the possibility to confirm granulomas with a minimally invasive diagnostic procedure (e.g., biopsy of skin lesions or superficial lymph nodes), patients with Lofgren syndrome, inability to undergo endoscopy, pregnancy, or inability to consent. | 304* | 8.0% | Percentage of bleeding in patients undergoing bronchoscopy. *149 undergoing bronchoscopy. |
| | 66 | Bilaceroglu | Monaldi Archives for Chest Disease | 1997 | Endobronchial lung cancer patients | Included: Visible cauliflower-like or infiltrative endobronchial tumours were enrolled. Excluded: Patients with only extrinsic compression, extrabronchial tumour or submucosal/peribronchial involvement. | 151 | 7.0%-17.0% -13.0% | EBNA-forceps biopsy-bronchial brushing. |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 67 | Diette | Chest | 1999 | Lung transplant recipients (LTRs), and patients referred to bronchoscopy | Excluded: Patients who were mechanically ventilated, non-English speaking or had communication deficits that precluded answering questions, and who died within 48 hours of the procedure. | 697 | 44.5% - 17.5% | LTRs - other patients. |
| | 68 | Hetzel | The European respiratory journal | 2012 | Patients with suspected endobronchial tumours | Included: Patients with suspected endobronchial lesion based on clinical signs and radiological images, aged >18 years, who signed informed consent. Excluded: Patients with a bleeding diathesis, patients on anticoagulants, patients with SaO2<90% (under delivery of 2L O2/min), or unstable angina pectoris, myocardial infarction in the past month, or decompensated heart failure. | 593 | 69.4% (17.8%) | (Requiring intervention). |
| | 69 | Khan | Respiration | 2010 | Patients with visible endobronchial lesions. | Excluded: Patients aged <18 years, patients with anticoagulantia use, known bleeding diathesis, blood pressure >140/90 mmHg, pacemaker or defibrillator implants, poor cardiopulmonary reserve or marked hypoxaemia at rest, patients unable to cooperate, and patients enrolled previously in the study. | 160 | 84.4%- 15.6%-0.0% | Mild-moderate-severe. |
| | 70 | Williams | Chest | 1998 | Consecutive patients referred to bronchoscopy | Excluded: Patients with tracheostomy, cerebral secondaries, supraventricular tachycardia, haemoptysis, unstable angina and mechanical ventilation. | 100 | 42.0% - 48.0% - 10.0% | Without need for wash - needed saline wash - needed adrenalin. |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 71 | Choi | The International journal of tuberculosis and lung disease | 2005 | Adult, Korean patients, referred to bronchoscopy | Excluded: Patients subject to follow-up after lung resection surgery, patients receiving midazolam, therapeutic bronchoscopy, patients without an interview due to early discharge, patients experiencing lidocain toxicity, patients unable to speak Korean, and patients who refused to enroll in the study. | 307 | 43.0-35.6% | Nasal - oral insertion of scope. |
| ***Pneumothorax*** | | | | | | | | | |
| | 55 | D'Ippolito | Monaldi Archives for Chest Disease | 2007 | Elderly patients | Excluded: Platelet count <50 000, APTT >=50 s or PTT =<75%, and haemodynamic instability. | 301 | 0.3% | Occurred in one patient without a biopsy procedure |
| | 57 | Dang | Internal medicine journal | 2012 | Consecutive patients referred to bronchoscopy | Excluded: Patients with hypersensitivity or allergies to anaesthetic medications, opioids or benzodiazepines, with brady- or tachycardia (resting heart rate <60 or >100 bpm), or systolic blood pressure of <100 or >180 mmHg), concurrent psychological disorders, those already on high doses of opioids or benzodiazepines, and oxygen requirements of >4 L/min O2 at rest. | 539 | 0.5% - 1.6% | Direct consequence of FFB - percentage of TBB. |
| | 58 | Jain | Chest | 2004 | Immunocompromised patients with lung infiltrates | Included: Immunocompromised patients with the presence of either a focal or diffuse pulmonary infiltrate, referred and giving informed consent to bronchoscopy. Excluded: HIV positive patients, lung transplant recipients. | 104 | 4 % | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 64 | Herth | Chest | 2002 | Patients aged > 40 undergoing TBB | Excluded: Patients with warfarin/heparin use last 2 weeks, known bleeding disorder, or TPK<80 000cells/mcL. | 1217 | 2.1% | |
| | 65 | von Bartheld | JAMA | 2013 | Patients with sarcoidosis | Excluded: Patients with obvious organ involvement of sarcoidosis with the possibility to confirm granulomas with a minimally invasive diagnostic procedure (biopsy of skin lesions or superficial lymph nodes), patients with Lofgren syndrome, inability to undergo endoscopy, pregnancy, or inability to consent. | 304* | 0.7% | One patient, after TBB. * 149 undergoing bronchoscopy. |
| | 66 | Bilaceroglu | Monaldi Archives for Chest Disease | 1997 | Endobronchial lung cancer patients | Included: Visible cauliflower-like or infiltrative endobronchial tumour patients. Excluded: Patients with only extrinsic compression, extrabronchial tumour or submucosal/peribronchial involvement. | 151 | 0.0% | |
| | 72 | Hirose | Respirology | 2008 | Japanese, adult patients undergoing diagnostic bronchoscopy | Excluded: Patients with known confusion (cerebral metastases, degenerative dementia, or other conditions), or patients judged too ill to participate, intubation, aged >80 years, patients unable to speak Japanese. | 129 | 0.0% | |
| | 73 | Tukey | Respiratory medicine | 2012 | Population based | Excluded: Patients who had undergone invasive procedures associated with complications during the same visit. | - | 0.97% | Biopsy procedures complicated by pneumothorax. |
| ***Bronchospasm*** | | | | | | | | | |
| | 55 | D'Ippolito | Monaldi Archives for Chest Disease | 2007 | Elderly patients | Excluded: Platelet count <50 000, APTT >=50 s or PTT =<75%, and haemodynamic instability. | 301 | 1.1% | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 57 | Dang | Internal medicine journal | 2012 | Consecutive patients referred to bronchoscopy | Excluded: Patients with hypersensitivity or allergies to anaesthetic medications, opioids or benzodiazepines, with brady- or tachycardia (resting heart rate <60 or >100 bpm), or systolic blood pressure of <100 or >180 mmHg), concurrent psychological disorders, those already on high doses of opioids or benzodiazepines, and oxygen requirements of >4 L/min O2 at rest. | 539 | 0.0% | |
| | 74 | Kaur | Chest | 2015 | Consecutive patients referred to bronchoscopy | Included: Patients with indication for flexible bronchoscopy, aged 12 to 90 years, who had systolic BP 100 - 180 mm Hg. Excluded: Pregnant patients, SaO2<92% with FiO2 <0.3, patients undergoing TBNA or other interventions, patients failing to provide informed consent. | 500 | 0.0% | Bronchospasm. |
| | 75 | Tapanainen | Respiratory medicine | 2002 | Asthmatics | Included: Non-smoking 18-60 year-old asthmatics[ATS criteria (5)] FEV1 from 60-100% of predicted and a moderate or severe bronchial hyperreactivity [provocative dose of histamine inducing a fall of 15% in FEV1 less than 0,4 and 0,1 mg respectively. | 57 | 12.0% | Cough/Bronchospasm that interfered with procedure. |
| ***Hypoxaemia*** | | | | | | | | | |
| | 59 | Grendelmeier | Swiss Medical Weekly | 2011 | Elective bronchoscopy patients | Excluded: Patients who were intubated and patients with known propofol allergy/intolerance. | 440 | 16.4% | Fall in SaO2 to <90%. |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 61 | Grendelmeier | The European respiratory journal | 2014 | Patients undergoing non-emergency bronchoscopy | Excluded: ICU/emergency bronchoscopy, patients aged <18 years, intubated or isolated patients, patients with known propofol allergy/intolerance, pregnant or breastfeeding patients, and patients with a mental disorder preventing appropriate judgment concerning study participation. | 702 | 28.9% | Fall in SaO2 to <90%. |
| | 63 | Ni | Chang Gung medical journal | 2010 | Patients undergoing diagnostic bronchoscopy | Excluded: patients aged <18 years, ASA IV or V, neurologic disorders or other conditions contributing to difficulty in assessing a conscious response, FVC < 15 ml/kg body weight, FEV1 < 1000 ml, or FEV1/FVC < 35%, patients with known allergy to study drugs, and patients with glaucoma. | 88 | 4.5% - 31.8% | Fall in SaO2 to <90%. Unsedated - Sedated patients. |
| | 76 | Lo | PLoS One | 2011 | Elective bronchoscopy patients | Exluded: Patients aged <18 years, ASA IV or V, neurologic disorders, FVC < 15 ml/kg, FEV1 < 1L, FEV1/FVC < 35%, Mallampati score of 4. | 492 | 37.8% | Fall in SaO2 to <90%. |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 77 | Ogawa | Respiratory investigation | 2014 | Consecutive patients ≥ 20 years undergoing diagnostic bronchoscopy | Included: Patients able to answer a simple questionnaire without assistance; adequate hepatic function (AST≤100 IU/L, ALT≤100 IU/L, and total bilirubin≤1.5 mg/dL); adequate renal function (serum creatinine≤1.5 mg/dL); and adequate respiratory function (PaO2≥60 mmHg or SaO2≥90% while breathing room air). Excluded: Patients with type II chronic respiratory failure requiring LTOT; sleep apnea syndrome requiring CPAP; history of severe drug allergy; neuromuscular disorders; acute narrow-angle glaucoma; myocardial infarction onset within 6 weeks; and pregnant or lactating women. | 204 | 75.5% | Percentage in need of supplemental oxygen to prevent SpO2 from declining to <90%. |
| | 78 | Fruchter | Respiration | 2014 | Elective bronchoscopy patients | Excluded: Patients unable to give or who refused to provide informed consent, patients aged <18 years, patients with endotracheal tube or tracheostomy, and patients with propofol allergy. | 81 | 74.0% | Patients who required supplemental oxygen by 100% O2 mask due to hypoxaemia (SaO2<90%). |
| | 79 | Ryu | British journal of anaesthesia | 2012 | Elective bronchoscopy patients | Included: Patients aged 18-70 years, ASA I-III. Excluded: Patients with SaO2 <90%, FEV1 < 1.0L, HR < 60 bpm, systolic pressure <100 mmHg, asthmatics, COPD patients, and pregnant patients. | 72 | 28.6% - 2.9% | Oxygen desaturation (<90% for >10s): propofol–dexmedetomidine group - propofol–remifentanil group. |

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|---|---|---|---|---|---|---|---|---|---|
| | 80 | Rosell | Respiratory medicine | 2006 | Consecutive, adult patients referred to bronchoscopy with BAL | Contraindications for BAL: Lack of patient cooperation, hypercapnia/hypoxaemia uncorrected by supplemental O2 administration, serious cardiac arrhythmias, recent acute myocardial infarction or recent asthma attack, uncorrected bleeding diathesis and haemodynamic instability. | 295 | 3.4% | Fall in SaO2 to <90%. |
| | 81 | Gibson | Australian and New Zealand journal of medicine | 1990 | Patients undergoing BAL | Data was only included from patients whose transcutaneous PO2 was within + 5mmHg of the arterial PaO2 value. | 88 | 25.0% - 75.0% | Patients receiving oxygen - patients who did not receive oxygen. |
| | 82 | Stolz | Chest | 2005 | Patients undergoing diagnostic bronchoscopy | Excluded: Intubated patients, those receiving propofol (e.g. IV drug abuse), and patients requiring endobronchial ultrasound examination. | 150 | 4.0% - 8.0% | Nebulised lidocain - nebulised placebo, fall in SaO2 to <90%. |
| ***Haemodynamic variations*** | | | | | | | | | |
| | 59 | Grendelmeier | Swiss Medical Weekly | 2011 | Elective bronchoscopy patients | Excluded: Patients who were intubated and patients with known propofol allergy/intolerance. | 440 | 37.0% - 15.4% | Drop in SBP >20 mmHg – Hypotension. |
| | 61 | Grendelmeier | The European respiratory journal | 2014 | Patients undergoing non-emergency bronchoscopy | Excluded: ICU/emergency bronchoscopy, patients aged <18 years, intubated or isolated patients, patients with known propofol allergy/intolerance, pregnant or breastfeeding patients, and patients with a mental disorder preventing appropriate judgment concerning study participation. | 702 | 28.9% | Hypotension. |

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|---|---|---|---|---|---|---|---|---|---|
| | 63 | Ni | Chang Gung medical journal | 2010 | Patients undergoing diagnostic bronchoscopy | Excluded: patients aged <18 years, ASA IV or V, neurologic disorders or other conditions contributing to difficulty in assessing a conscious response, FVC < 15 ml/kg body weight, FEV1 < 1000 ml, or FEV1/FVC < 35%, patients with known allergy to study drugs, and patients with glaucoma. | 88 | 4.5% - 47.7% vs 20.5% | Hypotension-Hypertension in non-sedated vs sedated patients. |
| | 70 | Williams | Chest | 1998 | Consecutive patients referred to bronchoscopy | Excluded: Patients with tracheostomy, cerebral secondaries, supraventricular tachycardia, haemoptysis, unstable angina and mechanical ventilation. | 100 | | |
| | 74 | Kaur | Chest | 2015 | Consecutive patients referred to bronchoscopy | Included: Patients with indication for flexible bronchoscopy, aged 12 to 90 years, who had systolic BP 100 - 180 mm Hg. Excluded: Pregnant patients, SaO2<92% with FiO2 <0.3, patients undergoing TBNA or other interventions, patients failing to provide informed consent. | 500 | 0 % | Arrhythmias. Post-procedural increase in HR, RR, and BP (rates not provided). |
| | 76 | Lo | PLoS One | 2011 | Elective bronchoscopy patients | Exluded: Patients aged <18 years, ASA IV or V, neurologic disorders, FVC < 15 ml/kg, FEV1 <1L, FEV1/FVC < 35%, Mallampati score of 4. | 492 | 3.0% - 5.9% | MAP<60mmHg - SBP <90 mmHg. |
| | 78 | Fruchter | Respiration | 2014 | Elective bronchoscopy patients | Excluded: Patients unable to give or who refused to provide informed consent, patients aged <18 years, patients with endotracheal tube or tracheostomy, and patients with propofol allergy. | 81 | 16.0%- 6.2% | Hypotension - Hypotension requiring phenylephrine. |

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|---|---|---|---|---|---|---|---|---|---|
| | 79 | Ryu | British journal of anaesthesia | 2012 | Elective bronchoscopy patients | Included: Patients aged 18-70 years, ASA I-III. Excluded: Patients with SaO2 <90%, FEV1 < 1.0L, HR < 60 bpm, systolic pressure <100 mmHg, asthmatics, COPD patients, and pregnant patients. | 72 | 10.0%- 11.0% - 2.9% - 25.7% - 0.0% | Arrhythmias - Hypertension - Hypotension - Tachycardia – Bradycardia. |
| | 83 | Silvestri | Chest | 2009 | Patients undergoing bronchoscopy and meeting RCT criteria | Included: Patients aged ≥ 18 years, ASA I-IV, non-pregnant. Excluded: Patients with hypersensitivity to any anaesthetic or opioid; failure to meet non per os status; an abnormal, clinically significant ECG finding; participation in an investigational drug study within the previous month; patients with Mallampati score of IV, or Mallampati score of III and a thyromental distance of ≤ 4 cm, or a difficult airway for any other reason as judged by the clinician. | 252 | 3.2% - 0.0% | Hypotension - Bradycardia |
| ***Fever and infection*** | | | | | | | | | |
| | 55 | D'Ippolito | Monaldi Archives for Chest Disease | 2007 | Elderly patients | Excluded: Platelet count <50 000, APTT >=50 s or PTT =<75%, and haemodynamic instability. | 301 | 10.0% | Fever (axillary body temperature >=38degr. C). |
| | 57 | Dang | Internal medicine journal | 2012 | Consecutive patients referred to bronchoscopy | Excluded: Patients with hypersensitivity or allergies to anaesthetic medications, opioids or benzodiazepines, with brady- or tachycardia (resting heart rate <60 or >100 bpm), or systolic blood pressure of <100 or >180 mmHg), concurrent psychological disorders, those already on high doses of opioids or benzodiazepines, and oxygen requirements of >4 L/min O2 at rest. | 539 | 5.7% | Perceived fever within 48 hours. |

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|---|---|---|---|---|---|---|---|---|---|
| | 65 | von Bartheld | JAMA | 2013 | Patients with sarcoidosis | Excluded: Patients with obvious organ involvement of sarcoidosis with the possibility to confirm granulomas with a minimally invasive diagnostic procedure (biopsy of skin lesions or superficial lymph nodes), patients with Lofgren syndrome, inability to undergo endoscopy, pregnancy, or inability to consent. | 304* | 4.0% | Bronchosopy subjects developing temperature >39 degr. C. * 149 undergoing bronchoscopy |
| | 77 | Ogawa | Respiratory investigation | 2014 | Consecutive patients ≥20 years undergoing diagnostic bronchoscopy | Included: Patients able to answer a simple questionnaire without assistance; adequate hepatic function (AST≤100 IU/L, ALT≤100 IU/L, and total bilirubin≤1.5 mg/dL); adequate renal function (serum creatinine≤1.5 mg/dL); and adequate respiratory function ($PaO_2$≥60 mmHg or $SaO_2$≥90% while breathing room air). Excluded: Patients with type II chronic respiratory failure requiring LTOT; sleep apnea syndrome requiring CPAP; history of severe drug allergy; neuromuscular disorders; acute narrow-angle glaucoma; myocardial infarction onset within 6 weeks; and pregnant or lactating women. | 204 | 1.5% - 1.9% | Pneumonia – Fever. |
| | 84 | Meduri | Chest | 1991 | Immunosuppressed patients with diffuse pulmonary infiltrates | Included: Patients with cancer treated with chemotherapy, patients receiving steroids, bone marrow transplant recipients and patients with, or at high risk of developing, AIDS. | 52 | 33.0% | Temperature elevation 1 degr. C above baseline in patients undergoing BAL. |

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|---|---|---|---|---|---|---|---|---|---|
| | 85 | Krause | American Journal of Respiratory and Critial Care Medicine | 1997 | Consecutive patients referred to bronchoscopy | Excluded: Patients with fever or with infectious pneumonia and patients receiving more than 10 mg prednisolone equivalent per day. | 50 | 24.0% | Body temperature above 38 degr.C. No difference between BAL and non-BAL groups. |
| | 86 | Pereira | American Review of Respiratory Disease | 1975 | Hospitalised patients with indication for bronchoscopy | Excluded: Patients who had fever during the week before endoscopy; 5 procedures performed in 5 patients were excluded because of incomplete data. | 95 | 6.3% - 9.5% | Fever with-without infiltration on chest X-ray. |
| | 87 | Gonzalez Aguirre | The International journal of Tuberculosis and Lung Disease | 2015 | Hospitalised patients with indication for bronchoscopy | Included: Patients aged>18 years, with an indication for bronchoscopy. Excluded: Patients with tracheostomy, endotracheal intubation or contraindication for bronchoscopy (contraindications not listed). | 63 | 65.1% | Increase in symptomatology, mainly due to fever. Details not given. |
| | 88 | Yigla | The European respiratory journal | 1999 | Consecutive patients referred to bronchoscopy | Excluded: Patients with current respiratory tract infection or febrile illnesses and patients receiving antibiotic therapy within a week prior to the bronchoscopy. | 200 | 6.5% | Bacteriaemia rate. |
| ***Health care utilization*** | | | | | | | | | |
| | 53 | Dunagan | Chest | 1997 | Bone marrow recipients | Excluded: Patients whose clinical assessment indicated that oxygen and haemodynamic status might be compromised by the procedure. | 71 | 4.2% - 1.4% | Needed intubation - needed pressor treatment. |
| | 54 | Shannon | Bone Marrow Transplantation | 2010 | Bone marrow recipients | Included: Patients whose SaO2 exceeded 90% on room air or supplemental O2. For TBB with BAL: platelet count > 80 x106 /mcgL. | 501 | 0.6% | Percentage of patients who needed high inspired oxygen. |
| | 55 | D'Ippolito | Monaldi Archives for Chest Disease | 2007 | Elderly patients | Excluded: Platelet count <50 000, APTT >=50 s or PTT =<75%, and haemodynamic instability. | 301 | 0.0% | Reports no need for mechanical ventilation and no procedure interrupted by major complications. |

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|---|---|---|---|---|---|---|---|---|---|
| | 56 | Diaz-Guzman | Respiration | 2009 | Patients with pulmonary hypertension | Included: Patients with a diagnosis of PH defined as (1) mean PAP >25 mmHg measured by right heart catheterization or (2) right ventricular systolic pressure >40 mm Hg estimated by Doppler echocardiography and clinical evidence of heart failure. Controls: Patients that did not meet criteria for PH + underwent bronchoscopy by the same physician within 48 h of the study patients. | 90 | 0.0% | Reports no moderate or severe haemorrhage, refractory hypotension, haemodynamic instability, death, hospitalisation or respiratory failure. |
| | 57 | Dang | Internal medicine journal | 2012 | Consecutive patients referred to bronchoscopy | Excluded: Patients with hypersensitivity or allergies to anaesthetic medications, opioids or benzodiazepines, with brady- or tachycardia (resting heart rate <60 or >100 bpm), or systolic blood pressure of <100 or >180 mmHg), concurrent psychological disorders, those already on high doses of opioids or benzodiazepines, and oxygen requirements of >4 L/min O2 at rest. | 539 | 0.4% | Two patients: Pseudoseizure in need of neurological consultation, and one ventricular tachycardia in pre-existing severe cardiomyopathy. |
| | 58 | Jain | Chest | 2004 | Immunocompromised patients with lung infiltrates | Included: Immunocompromised patients with the presence of either a focal or diffuse pulmonary infiltrate, referred and giving informed consent to bronchoscopy. Excluded: HIV positive patients, lung transplant recipients. | 104 | 2.9% | Short-term ventilatory support after FFB (respiratory failure) in two patients. One patient required transfusion with packed RBCs. |
| | 59 | Grendelmeier | Swiss Medical Weekly | 2011 | Elective bronchoscopy patients | Excluded: Patients who were intubated and patients with known propofol allergy/intolerance. | 440 | 0.0% | Defined adverse events. |

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|---|---|---|---|---|---|---|---|---|---|
| | **60** | **Schlatter** | **The European respiratory journal** | **2011** | **Elective bronchoscopy patients** | **Excluded: Intubated patients, patients receiving interventional bronchoscopy (e.g. laser or stent), patients with known hydrocodone or propofol allergy/intolerance.** | **300** | **31 %** | **Complications in need of intervention.** |
| | 61 | Grendelmeier | The European respiratory journal | 2014 | Patients undergoing non-emergency bronchoscopy | Excluded: ICU/emergency bronchoscopy, patients aged <18 years, intubated or isolated patients, patients with known propofol allergy/intolerance, pregnant or breastfeeding patients, and patients with a mental disorder preventing appropriate judgment concerning study participation. | 702 | 0.3% - 0.3% - 6.3% | Patients transferred to ICU - intubated - receiving oro-/nasopharyngeal airway. |
| | 65 | von Bartheld | JAMA | 2013 | Patients with sarcoidosis | Excluded: Patients with obvious organ involvement of sarcoidosis with the possibility to confirm granulomas with a minimally invasive diagnostic procedure (biopsy of skin lesions or superficial lymph nodes), patients with Lofgren syndrome, inability to undergo endoscopy, pregnancy, or inability to consent. | 304* | 1.3% | Pneumothorax after TBB requiring chest tube drainage in one patient. Non-invasive ventilation (<12 hours) after general anaesthesia in one patient. |
| | 68 | Hetzel | The European respiratory journal | 2012 | Patients with suspected endobronchial tumours | Included: Patients with suspected endobronchial lesion based on clinical signs and radiological images, aged >18 years, who signed informed consent. Excluded: Patients with a bleeding diathesis, patients on anticoagulants, patients with SaO2<90% (under delivery of 2L O2/min), or unstable angina pectoris, myocardial infarction in the past month, or decompensated heart failure. | 593 | 17.8% | All interventions related to bleeding. |

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|---|---|---|---|---|---|---|---|---|---|
| | 70 | Williams | Chest | 1998 | Consecutive patients referred to bronchoscopy | Excluded: Patients with tracheostomy, cerebral secondaries, supraventricular tachycardia, haemoptysis, unstable angina and mechanical ventilation. | 100 | 0.0% | No admissions of outpatients. |
| | 73 | Tukey | Respiratory medicine | 2012 | Population based | Excluded: Patients who had undergone invasive procedures associated with complications during the same visit. | - | 0.55% | Pneumothorax after transbronchial biopsy requiring chest tube placement. |
| | 84 | Meduri | Chest | 1991 | Immunosuppressed patients with diffuse pulmonary infiltrates | Included: Patients with cancer treated with chemotherapy, patients receiving steroids, bone marrow transplant recipients and patients with, or at high risk of developing, AIDS. | 52 | 3.9% | Two patients experienced respiratory failure and were treated with mechanical ventilation. |
| | 86 | Pereira | American Review of Respiratory Disease | 1975 | Hospitalised patients with indication for bronchoscopy | Excluded: Patients who had fever during the week before endoscopy; 5 procedures performed in 5 patients were excluded because of incomplete data. | 95 | 1.0% | One patient needed antimicrobial therapy. |
| ***Coughing*** | | | | | | | | | |
| | 57 | Dang | Internal medicine journal | 2012 | Consecutive patients referred to bronchoscopy | Excluded: Patients with hypersensitivity or allergies to anaesthetic medications, opioids or benzodiazepines, with brady- or tachycardia (resting heart rate <60 or >100 bpm), or systolic blood pressure of <100 or >180 mmHg), concurrent psychological disorders, those already on high doses of opioids or benzodiazepines, and oxygen requirements of >4 L/min O2 at rest. | 539 | 10.8% | New cough after bronchoscopy. |

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|---|---|---|---|---|---|---|---|---|---|
| | 63 | Ni | Chang Gung medical journal | 2010 | Patients undergoing diagnostic bronchoscopy | Excluded: patients aged <18 years, ASA IV or V, neurologic disorders or other conditions contributing to difficulty in assessing a conscious response, FVC < 15 ml/kg body weight, FEV1 < 1000 ml, or FEV1/FVC < 35%, patients with known allergy to study drugs, and patients with glaucoma. | 88 | 31.1%-55.7% | procedures interfered by cough -patients with post-procedural cough |
| | 65 | von Bartheld | JAMA | 2013 | Patients with sarcoidosis | Excluded: Patients with obvious organ involvement of sarcoidosis with the possibility to confirm granulomas with a minimally invasive diagnostic procedure (biopsy of skin lesions or superficial lymph nodes), patients with Lofgren syndrome, inability to undergo endoscopy, pregnancy, or inability to consent. | 304* | 4.7% | Intolerable cough during bronchoscopy. *149 undergoing bronchoscopy. |
| | 71 | Choi | The International journal of tuberculosis and lung disease | 2005 | Adult, Korean patients, referred to bronchoscopy | Excluded: Patients subject to follow-up after lung resection surgery, patients receiving midazolam, therapeutic bronchoscopy, patients without an interview due to early discharge, patients experiencing lidocain toxicity, patients unable to speak Korean, and patients who refused to enroll in the study. | 307 | 44.3%-33.6% | Nasal - oral insertion of scope. Patient-reported |
| | 72 | Hirose | Respirology | 2008 | Japanese, adult patients undergoing diagnostic bronchoscopy | Excluded: Patients with known confusion (cerebral metastases, degenerative dementia, or other conditions), or patients judged too ill to participate, intubation, aged > 80 years, patients unable to speak Japanese. | 129 | 86.0% | Percentage of patients who reported to be "bothered by coughing" during bronchoscopy. |

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|---|---|---|---|---|---|---|---|---|---|
| | 75 | Tapanainen | Respiratory medicine | 2002 | Asthma patients | Included: Non-smoking 18-60 year old asthmatics (ATS criteria (5)). FEV1 from 60-100% of predicted and a moderate or severe bronchial hyperreactivity (provocative dose of histamine inducing a fall of 15% in FEV1 less than 0,4 and 0,1 mg respectively). | 57 | 52%-40%-12% | Cough-cough of no significance-cough/bronchospasm that interfered with procedure. |
| ***Other respiratory symptoms and signs*** | | | | | | | | | |
| | 57 | Dang | Internal medicine journal | 2012 | Consecutive patients referred to bronchoscopy | Excluded: Patients with hypersensitivity or allergies to anaesthetic medications, opioids or benzodiazepines, with brady- or tachycardia (resting heart rate <60 or >100 bpm), or systolic blood pressure of <100 or >180 mmHg), concurrent psychological disorders, those already on high doses of opioids or benzodiazepines, and oxygen requirements of >4 L/min O2 at rest. | 539 | 5.7% | Dyspnoea |
| | 61 | Grendelmeier | The European respiratory journal | 2014 | Patients undergoing non-emergency bronchoscopy | Excluded: ICU/emergency bronchoscopy, patients aged <18 years, intubated or isolated patients, patients with known propofol allergy/intolerance, pregnant or breastfeeding patients, and patients with a mental disorder preventing appropriate judgment concerning study participation. | 702 | 0.1% | One patient experienced respiratory failure. |

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|---|---|---|---|---|---|---|---|---|---|
| | 71 | Choi | The International journal of tuberculosis and lung disease | 2005 | Adult, Korean patients, referred to bronchoscopy | Excluded: Patients subject to follow-up after lung resection surgery, patients receiving midazolam, therapeutic bronchoscopy, patients without an interview due to early discharge, patients experiencing lidocain toxicity, patients unable to speak Korean, and patients who refused to enroll in the study. | 307 | 38.2% - 30.9% | Complained about dyspnoea: Nasal - oral insertion of scope. |
| | 75 | Tapanainen | Respiratory medicine | 2002 | Asthmatics | Included: Non-smoking 18-60 year-old asthmatics[ATS criteria (5)] FEV1 from 60-100% of predicted and a moderate or severe bronchial hyperreactivity [provocative dose of histamine inducing a fall of 15% in FEV1 less than 0,4 and 0,1 mg respectively. | 57 | 3.5% - 5.2% | Dyspnoea - Increase in asthma symptomatology |
| | 92 | Humbert | Thorax | 1996 | Asthma patients and controls | Excluded: Patients taking oral corticosteroids in the last three months, current smokers or ex-smokers with more than 5 pack years. Study 1: Excluded inhaled corticosteroids for the last three months. Study 2: Excluded inhaled corticosteroids for the last two weeks, patients aged <18 or >55 years, FEV1<50% of predicted, acute or chronic infection, pregnancy, breast feeding, or any other significant medical condition. | 77 | 4.7% - 2.9% | Experienced mild symptomatic wheeze after the bronchoscopic examination, which was rapidly reversed with an additional dose of salbutamol by nebulizer: Study 1 - study 2. No significant changes in daily asthma symptoms, rescue salbutamol therapy, and PEFR for two weeks before and after bronchoscopy. |
| ***Identified discomfort and pain*** | | | | | | | | | |
| | 89 | Webb | Thorax | 1990 | Patients undergoing diagnostic bronchoscopy | Excluded: Patients undergoing bronchoscopy for haemoptysis with a normal chest radiograph, stridor, abnormal coagulation, HIV positive. | 62 | | No difference in discomfort between groups receiving topical anaesthesia with two different techniques. |

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|---|---|---|---|---|---|---|---|---|---|
| | 90 | Watts | Respiratory medicine | 2005 | Elderly patients | Excluded: Patients taking any benzodiazepine/opioid, patients with a resting SaO2 <90% on room air, an abbreviated Mental Test Score < 7/10 or those unable to perform a VAS. | 50 | | Patient derived VAS scores of discomfort and willingness to return were significantly lower (better) in the group treated with oral temazepam plus nebulised lidocaine than in the alfentanyl group. |
| | 91 | Stolz | Thorax | 2004 | Patients undergoing diagnostic bronchoscopy | Excluded: Intubated patients. | 110 | | The patients' tolerance was significantly better in the midazolam + hydrocodon group compared to the midazolam + placebo group. |
| | 93 | Diette | Chest | 2003 | Consecutive patients referred to bronchoscopy | Included: Patients aged >18 years undergoing bronchoscopy. Excluded: Non-English-speakers, patients with encephalopathy or other significant alteration of mental status, sensory deficits that precluded the use of a visual and auditory aid, or contact isolation. | 80 | | |
| | 94 | Putinati | Chest | 1999 | Patients undergoing a routine diagnostic FFB for the first time | Excluded: Patients undergoing BAL and TBB, as well as intubated patients. | 100 | | The tolerance score attributed by the patients was better in the diazepam sedation group than in the non-sedation group ($p<0.05$). FFB was better tolerated in male patients ($p< 0.05$), in patients with less pre-FFB anxiety score, and in the diazepam sedation group ($p< 0.01$). |

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|---|---|---|---|---|---|---|---|---|---|
| | 95 | Yoon | Acta anaesthesiologica Scandinavica | 2011 | Elective bronchoscopy patients | Excluded: Patients aged >75 years, patients with asthma or FEV1<1.0 L, patients who had resting hypoxaemia and needed supplementary oxygen in the resting state, patients with a history of alcohol abuse or current use of any psychiatric medication, and patients refusing to give informed consent. | 64 | | There were no differences in the satisfaction VAS scores between the propofol and propofol + alfentanil group. |
| | 96 | Hwang | Acta anaesthesiologica Scandinavica | 2005 | Patients undergoing diagnostic bronchoscopy | Excluded: Patients allergic to study medication, those judged unable to use the Patient-controlled-sedation system, and patients who had an endotracheal tube or tracheostomy. | 264 | | Patients in the propofol + ketamine group were more satisfied with the degree of sedation than those in the propofol + alfentanyl group (9.5 (6–10) vs. 9.0 (6–10), $P < 0.05$). The satisfaction score attributed by the bronchoscopists was not significantly different in the two groups. |
| | 97 | Schwarz | Respiration | 2007 | Consecutive patients referred to bronchoscopy | Excluded: Patients aged <18 years; pregnant patients; nursing women; patients allergic to opioids, patients with usage of opioids, sedatives or centrally acting drugs (central nervous system depressants or antidepressants) during 12 hours prior to bronchoscopy. | 59 | | The dextromethorphan sedation group had lower pain scores ($p<0.00005$), and the difference continued for 2 h post bronchoscopy ($p<0.05$). They had a significantly lower preoperative emotion score ($p<0.00001$), a significant reduction in the score of complaints after the procedure ($p<0.0001$), in the patients' evaluation of the stress level during the procedure ($p<0.005$), and in the level of fear of the procedure ($p<0.0005$), and felt that the procedure was much less unpleasant than they |

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|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | had expected ($p<0.00005$). |

Paper I

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REVIEW ARTICLE

# Complications and discomfort of bronchoscopy: a systematic review

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***Objective***: To identify bronchoscopy-related complications and discomfort, meaningful complication rates, and predictors.
***Method***: We conducted a systematic literature search in PubMed on 8 February 2016, using a search strategy including the PICO model, on complications and discomfort related to bronchoscopy and related sampling techniques.
***Results***: The search yielded 1,707 hits, of which 45 publications were eligible for full review. Rates of mortality and severe complications were low. Other complications, for instance, hypoxaemia, bleeding, pneumothorax, and fever, were usually not related to patient characteristics or aspects of the procedure, and complication rates showed considerable ranges. Measures of patient discomfort differed considerably, and results were difficult to compare between different study populations.
***Conclusion***: More research on safety aspects of bronchoscopy is needed to conclude on complication rates and patient- and procedure-related predictors of complications and discomfort.

Keywords: *diagnostic bronchoscopy*; *safety*; *adverse events*; *patient satisfaction*; *informed consent*

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Flexible bronchoscopy (FB) was introduced in 1968, and today it is an essential procedure in respiratory medicine. There are numerous indications for bronchoscopy, and it is frequently used for diagnostic and therapeutic purposes in both inpatients and outpatients. White light FB is commonly used in diagnostics, as it enables visualisation of the lower airways and sampling techniques such as bronchial brushings (BB), bronchial washings (BW), bronchoalveolar lavage (BAL), endobronchial biopsies (EBB), transbronchial biopsies (TBB), and transbronchial needle aspiration (TBNA) (1).

Bronchoscopy is generally considered safe (2). However, whether performed with anaesthesia or only light sedation, pre-procedural medications are routinely administered and may have side effects. Diagnostic sampling may lead to immediate, although rare, complications, such as intrabronchial bleeding, bronchospasm, and pneumothorax. In addition, some discomfort may be felt in the days after the procedure, such as fever, sore throat, cough, or reactions to the medications used (1). Events occurring after the observation period may not be detected by the bronchoscopist. To ensure that both the bronchoscopy team and the patient are adequately prepared for the procedure, a realistic picture of the potential for complications and discomfort is imperative.

To the best of our knowledge, there is no recent systematic review of complications and discomfort associated with bronchoscopy. The 2013 British Thoracic Society Guidelines (2) includes a comprehensive overview of complications, but only presents a few selected references without discussing potential weaknesses of the included studies.

Thus, we set out to conduct a systematic review of complications and patient discomfort associated with non-interventional bronchoscopy, and the frequency and predictors of these in patients and research subjects.

## Methods

We used a modified Population - Intervention - Outcome comparison (PICO) form (3) (Table 1) and performed



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*Table 1.* Search word combinations, in a modified PICO form for a systematic literature search on complications and discomfort related to bronchoscopy

| We are interested in a procedure called (Intervention 1) | Where ... is performed. (Intervention 2) | Will it lead to ...? (Outcome) |
|---|---|---|
| Bronchoscopy | Bronchoalveolar lavage | Complication[a] |
| | BAL | Discomfort |
| | Brush[a] | Cough[a] |
| | Transbronchial biopsy | Saturation decrease |
| | Endobronchial biopsy | Adverse events |
| | Bronchial biopsy | Adverse effects |
| | Conscious sedation | Bronchospasm |
| | Lidocaine | Death |
| | | Pneumothorax |
| | | Shortness of breath |
| | | Dyspnoea |
| | | Bleeding |
| | | Haemorrhage |
| | | Fever |
| | | Vasovagal syncope |
| | | Cardiac arrest |
| | | Contraindication |
| | | Safety |
| | | Patient experience |
| | | Adverse symptoms |
| | | Anxiety |
| | | Pain |
| | | Hospitalisation |

[a]Truncation. The content of columns was combined with OR. Different columns were combined with AND.

a systematic literature search in PubMed (Medline). Keywords were selected by combining existing thesauruses (MeSH terms) and text words. We performed a review of the existing MeSH database and of the (MeSH) classification of relevant papers that were already published. In addition, we added text words considered relevant to describe complications known to the authors.

The search in PubMed was conducted on 8 February 2016.

We included publications in English, Norwegian, Swedish, Danish, and French. Case reports, non-original research (letters, review articles, guidelines, etc.), animal studies, studies solely based on interventional procedures and specialised examination techniques, studies on paediatric populations as well as studies of intubated patients, patients on mechanical ventilation, under general anaesthesia or in an intensive care unit (ICU), were excluded, along with publications that did not cover the topic on complications or discomfort associated with bronchoscopy. Studies on bronchoscopes as a source of contamination were considered outside the scope of the current review.

Papers were classified as prospective or retrospective, and whether investigation of complications and discomfort was considered an objective (primary, secondary, not formalised). We also divided articles into three groups based on the number of subjects in the study and identified studies on medication during or before bronchoscopy.

Full review was only performed on papers where complications or discomfort was a primary or secondary objective of the study, where the number of subjects exceeded 50, and where there was given a sufficient description of the sample and the sampling methods (inclusion/exclusion criteria, definition of endpoints, and data collection). We chose to exclude papers based on less than 50 subjects since the statistical power of these studies in detecting rare complications is bound to be low.

## Results

The initial literature search yielded 1,707 papers, of which 1,435 were excluded (Table 2). In total, 94 papers reported complications and discomfort as their primary or

*Table 2.* Yield of a PubMed – literature search on discomfort and complications related to bronchoscopy (8 February 2016)

| | Number of articles |
|---|---|
| Total in search | 1,707 |
| Type of publications | |
| Excluded, non-original | 214 |
| Excluded, language | 183 |
| Excluded, case studies | 268 |
| Excluded, not human | 37 |
| Type of bronchoscopy | |
| Excluded, provocation test | 24 |
| Excluded, interventional bronchoscopy | 26 |
| Excluded, general anaesthesia/intubated/ mechanical ventilation/ICU | 149 |
| Excluded, endobronchial ultrasound (EBUS) | 32 |
| Excluded, experimental or non-standard bronchoscopy techniques | 7 |
| General | |
| Excluded, no relevance/does not address complications nor patient experience | 381 |
| Excluded, children | 110 |
| Excluded, disease outbreak study | 3 |
| Excluded, did not report according to objective[a] | 1 |
| Publications excluded, total | 1,435 |
| Publications remaining, total | 272 |

[a]One study did not report complications, despite the objective '(...) to document any complications'.



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secondary objective in procedures on more than 50 subjects (Table 3). Of these papers, 15 did not define outcomes sufficiently (4–18), five papers did not give information on the data collection (6, 7, 13, 14, 19), four papers were based on surveys of health care suppliers (20–23), and inclusion or exclusion criteria were not specified in 37 papers (8, 9, 12, 17, 20–52). Thus, further review was performed on the remaining 45 publications. The articles are subsequently reviewed with respect to the subtopics: death, bleeding, pneumothorax, bronchospasm, hypoxaemia, haemodynamic variations, fever and infection, health care utilisation, coughing, other respiratory symptoms and signs, and identified discomfort and pain The publications are further described in the Supplementary file.

### Death

Nine papers specified death as a potential outcome (53–61). The studies comprised 71–702 subjects (53, 61). All studies, except Grendelmeier et al. (59, 61), were conducted on selected populations (mostly immunocompromised individuals). As in all but one study (53), Grendelmeier et al. report a mortality rate of 0% (59, 61).

### Bleeding

Bleeding rates varied between 2.5 and 89.9% in the prospective studies and drug studies (59, 62). The studies comprised 88–1,217 subjects (63, 64). Some studies graded severity of bleeding according to volume (58, 62, 65–67), whereas others graded in terms of required intervention (63, 64, 68–70). Three studies did not define bleeding (59, 61, 71). Carr et al. aimed to investigate actual blood loss in 234 patients with low risk of bleeding. They categorised bleeding as minimal ( $<5$ ml), mild (5–20 ml), moderate (20–100 ml), and severe ( $>100$ ml) and found that 89.7% had minimal bleeding, 8.1% had mild bleeding, and 2.1% had moderate bleeding. No patients had severe bleeding. Superior vena cava syndrome and addition of EBB and TBB to TBNA predicted bleeding (62).

### Pneumothorax

Six prospective studies (57, 58, 64–66, 72) and two retrospective studies (55, 73) listed pneumothorax as a potential outcome, with rates ranging from 0 to 4% (58, 72). Two studies reported no pneumothoraces in various bioptic techniques that included TBB (66, 72). Jain et al. reported 4% pneumothorax but did not relate complications to the specific procedure (58). Dang et al. reported that pneumothorax occurred in three patients at a rate of 1.6% when expressed as a percentage of TBB. One pneumothorax required intervention (57). Herth et al. conducted a study on 1,217 patients going through TBB and found that 26 of them (2.1%) developed pneumothoraces, of which 14 were treated with tube thoracostomy, and the remaining 12 required no intervention (64). There were no prospective studies reporting pneumothorax as a result of other sampling procedures, such as brush sampling or lavage.

A large, retrospective population-based register study found that 0.97% (95% confidence interval (CI): 0.94–1.01%) of transbronchial lung biopsies were complicated by a pneumothorax that required chest tube placement (73).

### Bronchospasm

Three prospective studies (57, 74, 75) and one retrospective study with prospective recordings of bronchospasm (55) reported on bronchospasm. Bronchospasm occurred at a rate between 0 and 12.3% (57, 75). The rate of 12.3% was found in a study including asthma patients exclusively (75).

### Hypoxaemia

Ten studies provided information on hypoxaemia in unselected, elective patients (59, 61, 63, 76–82). The studies

*Table 3.* Quantitative overview of articles from a systematic literature search on complications and discomfort of bronchoscopy, divided into groups based on study design characteristics, number of subjects investigated, and relevance to the topic of complications and discomfort

| | Subjects | Primary objective | Secondary objective | Reports complication | Claims 'no complications' | Total |
|---|---|---|---|---|---|---|
| Prospective studies | $n>200$ | 14[a] | 3[a] | 4 | 0 | 21 |
| | $n$ 50–200 | 31[a] | 2[a] | 26 | 4 | 63 |
| | $n<50$ | 28 | 4 | 31 | 11 | 74 |
| Retrospective studies | $n>200$ | 15[a] | 4[a] | 12 | 3 | 34 |
| | $n$ 50–200 | 3[a] | 2[a] | 13 | 2 | 20 |
| | $n<50$ | 8 | 7 | 7 | 7 | 29 |
| Medication studies | $n>200$ | 5[a] | 0 | 0 | 0 | 5 |
| | $n$ 50–200 | 15[a] | 0 | 0 | 0 | 15 |
| | $n<50$ | 8 | 0 | 3 | 0 | 11 |
| Total | | 127 | 22 | 96 | 27 | 272 |

[a]In total, 94 articles reported complications and discomfort as their primary or secondary objective in procedures on more than 50 subjects.

comprised 73–702 subjects (61, 79). The majority of these prospective studies and drug studies defined hypoxaemia, or desaturation, as an oxygen saturation $\leq$90% (59, 61, 63, 76–80, 82) or as a drop in $pO_2$ to $<$60 mmHg at varying time points (81). The papers reported desaturation rates between 0.7 and 76.3% (80, 81). Rates around 75.0% (duration not defined) were observed in both subjects with (78) and without supplemental oxygen (77, 81). Fruchter et al. aimed at conscious sedation (propofol), which is defined as being able to rouse the patient by mild prodding or shaking (78). Grendelmeier et al. reported more mid-range results, with desaturation less than 90% in 16.4% of 440 patients going through bronchoscopy with propofol sedation, with conscious sedation defined as onset ptosis (59). Of note is that only two out of 10 studies specified a level of hypoxaemia at which they considered bronchoscopy contra-indicated (77, 79).

### Haemodynamic variations

Eight drug studies (61, 70, 74, 76, 78, 79, 82, 83) and two prospective studies (59, 63) reported haemodynamic complications. The studies comprised 72–702 subjects (61, 79). In six papers, hypotension was regarded as a systolic blood pressure (SBP) of $<$90 mmHg (59, 61, 63, 76, 78, 79) that required intervention (83). Hypotension ranged from 2.9%, in patients sedated with propofol and dexmedetomidine (79), to 28.9% in propofol sedation (61). Two papers reported that 1–16% of participants needed fluid resuscitation due to hypotension (78, 83). No paper reported clinical outcome associated with hypotension. Only two studies defined hypertension: one as SBP $>$ 180 mmHg or diastolic BP $>$90 mmHg (63) and the other as BP $>$140/90 (79). Bradycardia was defined in three studies, $<$60/min (79), $<$55/min (70), and $<$50/min, and required intervention (83). All reported the incidence of bradycardia to be 0. Two drug studies defined tachycardia, $>$100/min (79) and $>$130/min (70), and reported incidence rates of 25.7% (79) and 8.0% (70), respectively. Ryu reported 10.0% arrhythmias (79). Information regarding the need of anti-arrhythmic therapy was not given in any of these studies (70, 79).

### Fever and infection

Elevation of body temperature was reported in seven prospective studies (57, 65, 77, 84–87) and one retrospective study with prospective recordings of temperature (55). The studies comprised 50–539 subjects (57, 85). The range in incidence was 2–33% (77, 84). No studies used comparable definitions of 'fever' or 'temperature change'. Krause et al. defined fever as a rise in body temperature to $>38^{\circ}$C. Axillary body temperature was measured in the morning prior to bronchoscopy and 3, 6, 12, and 24 h after examination. In 20 patients, BAL was performed; 30 patients were examined by bronchoscopy only; 12 patients (24%) developed fever. There was no difference between the BAL and non-BAL groups (85). González Aguirre et al. reported an increased symptomatology in 65.1% post-FB and stated that this was mainly due to fever. The number of patients experiencing fever was not reported (87). Other signs, symptoms, and findings related to infection were reported in six prospective studies (75, 77, 85–88). Yigla et al. studied 200 patients without pre-procedural pulmonary infection and found a 6.5% of bacteraemia rate following bronchoscopy (88). In a study of asthma patients, 7% experienced respiratory infection during the 2 weeks following bronchoscopy, but antibiotic treatment or other required intervention was not reported (75). Krause et al. found flu-like symptoms in 8 out of 12 patients with fever, and two with chills and severe constitutional symptoms, all of whom responded well to Non Steroid Antiinflammatory Drugs (NSAIDs) and subsided within 24 h (85). Pereira et al. reported that one patient with protracted fever had a progressive pneumonitis with a fatal outcome following bronchoscopy despite antimicrobial drug therapy. All other cases of fever subsided without antimicrobial treatment (86).

### Health care utilisation

Nine prospective studies (57–59, 61, 65, 68, 70, 84, 86) reported complications that had to be handled by increased health care utilisation. Similarly, five retrospective studies (53–56, 73) reported events of increased healthcare utilisation. Tukey and Wiener used health care registers to identify pneumothoraces and haemorrhages coded as iatrogenic and subsequently attributed them to bronchoscopic procedures (73). The remaining studies comprised 71–702 subjects (53, 61). The incidence of health care contacts ranged from 0 to 31%, (59, 60) but was difficult to compare across different studies and designs. We were not able to conclude regarding admission rates, prolonged observation after bronchoscopy, or regarding assistance from outpatient/emergency room services after the initial in-hospital observation.

### Coughing

In some papers, coughing was referred to as a complication or adverse event (57, 59, 63, 65, 71, 75, 87, 89), and in others it was simply a measure of discomfort (61, 72, 74, 82, 90, 91). Six prospective studies, comprising 57–539 subjects (57, 75), reported cough by giving the proportion of patients who experienced or were bothered by coughing (57, 63, 65, 71, 72, 75). In these studies, the rate ranged from 4.7 to 86.0% (65, 72). Procedural cough was investigated in five articles (63, 65, 71, 72, 75). Post-procedural cough was investigated in two papers, with an incidence of 10.8% (57) and 55.7% (63). None of the above-mentioned papers reported on the duration of cough. Visual analogue scale (VAS), numeric rating scale (NRS), and cough counting were the main rating tools of cough in the drug trials; however, results were difficult to compare as they investigated different drug regimens and



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primarily reported differences in cough related to sedation or topical anaesthesia in subgroups within the trial.

### *Other respiratory symptoms and signs*

Papers reporting on respiratory symptoms besides cough and bronchospasm included five prospective studies (57, 61, 71, 75, 92). Two papers investigated change in asthma symptom scores in a 2-week period following bronchoscopy. Humbert et al. found no change in asthma score (92), whereas Tapanainen et al. found that 5.3% had an increase in asthma symptoms (75). Two papers reported rates of dyspnoea between 3.5% (75) and 5.7% (57) as observed by the researchers. In a study by Choi et al., self-reported shortness of breath was 38.2 and 30.9% in subgroups of nasal and oral insertion of bronchoscope, respectively (71). In other studies investigating patient-reported dyspnoea, rates were not possible to extract as only the ratios between subgroups were given in the papers (53–59, 61, 65, 68, 70, 73, 84, 86).

### *Identified discomfort and pain*

Eight prospective studies (63, 71, 72, 77, 87, 89, 93, 94) and 12 drug studies (60, 61, 74, 76, 79, 82, 83, 90, 91, 95–97) reported subjective measures of patient satisfaction or discomfort related to bronchoscopy. Numeric rating scales (NRS), verbal analogue scales (VAS) and visual analogue scales (VAS) were the most common assesment tools. Several different scales were employed: verbal analogue scales from 0 to 10 (63, 76): 10-point Likert scale (1–10) (83); VAS 0–10 cm (with opposite orientations) (60, 82, 87, 90, 91, 96, 97); VAS 0–100 mm (with opposite orientation) (72, 89, 95); NRS 0–100 (79); faces pain rating scale (0–5) (74); and grading distress as no, some, or extreme distress (77). Drug studies and studies evaluating different clinical interventions used these scales to compare the patient satisfaction between the intervention groups (60, 63, 74, 76, 79, 82, 83, 87, 89–91)(94–97). The only measure of satisfaction that was comparable between studies was 'willingness to return', which was used in six studies (61, 63, 71, 72, 79, 83) ranging between 55.4 and 96.3% (61, 71).

## Discussion

We have presented a systematic review on complications and discomfort of FB. Severe complications were rare; pneumothorax requiring intervention was reported in 0–2.1% of patients who had undergone TBB (64, 72). Mortality rate was low, but it was difficult to compare between studies that were performed on more or less selected populations. The willingness to repeat bronchoscopy was well above 50%.

Rates of specific complications ranged considerably, as in the case of oxygen desaturation [0.7–76.3% of patients (80, 81)] and bleeding (2.5–89.9% of patients) (59, 62). There are several potential reasons for this: the wide range of definitions (discussed below), different schemes for data collection, differences in equipment and techniques, differences between patient populations, and possibly time-dependent inter-study differences, as there are more than 40 years of gap between the publications included in this review. We argue that the considerable variability in complication rates can be attributed to a lack of consensus on how to define and measure complications and that many of the presented studies have a modest sample size.

Patient tolerance was difficult to assess as all studies utilised different measures of discomfort. VAS and NRS were mostly used to compare subgroups receiving different drug regimens, and it was unclear whether the results of these studies were representative for clinical practice.

Furthermore, absolute scale values were rarely presented in result sections, as relative comparison between subgroups was preferred.

The closest we got to a mortality rate that is representative of routine clinical practice was in one of the excluded studies. Facciolongo and co-workers reported a mortality rate of 0.02% in a large prospective study in 19 centres conducting diagnostic and therapeutic bronchoscopy. All deaths were somehow related to patients with a scheduled bronchoscopic laser treatment. This report was excluded from our main review because the authors did not specify how patients were selected for inclusion, and with regard to other complications they reported an unusual low number of incidents (1.08% of procedures) (24).

That we had to resolve to referring an excluded article when discussing a major outcome such as mortality illustrated one potential weakness of our approach – we might have applied much rigorous exclusion criteria. However, the informed reader needs to evaluate the external validity of the included studies, and we considered a comprehensive description of the sampling process as imperative for this purpose. We have also chosen to exclude more specific procedures such as bronchoscopy in the ICU, endobronchial ultrasound (EBUS), and interventional ultrasound, which should be topics of separate, future reviews.

Although bronchoscopy appeared to be a safe procedure in terms of mortality, bleeding, and pneumothoraces, it was difficult to conclude regarding the frequency of other specific complications. The inter-study variation in definitions of specific complications was considerable if the outcomes were defined at all. In particular, this could be exemplified by the variation in desaturation and bleeding rates, as well as cough, health care utilisation, and discomfort. The variation in definitions of 'complications' can have several reasons, but it is likely due to the researchers' and clinicians' perception of what can be considered significant complications, and which adverse events are relevant for a specific patient group. Definitions may also vary due to available tools for recording adverse events. We also observed a lack of studies addressing

complications and discomfort related to specific sampling techniques, sedation, duration of the procedure, and experience of the bronchoscopist. Similarly, there were few articles that reported patient characteristics related to safety and discomfort, such as indication for bronchoscopy, comorbidities, age, and pre-procedural anxiety. In the case of hypoxaemia, only two of the studies that provided desaturation rates specified a pre-procedural minimum resting/room air saturation of the participants ($>90\%$) (77, 79). Few subtopics in our article present predictors of complications, and we cannot, finally, conclude on predictors of complication. This is mainly due to predictors not being presented in the reviewed articles, which could result from insufficient statistical power.

## Conclusion

To conclude, bronchoscopy is a safe procedure in terms of complications such as mortality, pneumothorax, and bleeding that necessitate intervention. However, we should be able to inform patients in less broad strokes, with details concerning risk of both complications and what clinicians would characterise as discomforts. To provide this information, we need a sufficiently powered, prospective study on a well-described sample with clear definitions of complications that at least include mortality, pneumothorax, desaturation, bleeding, hypotension, arrhythmia, fever, and 'willingness to return'. Characteristics of participants and procedures should be related to the outcomes in order to identify high-risk procedures. In addition, all complications should be characterised in terms of necessary intervention.

## Authors' contributions

EOL, EMHM, TMLE, PSB, and RG took part in developing the research question. EOL, EMHM, and RG took part in the development of the search strategy, and EOL and RG systematised the publications and wrote the first draft. EOL, EMHM, TMLE, PSB, and RG critically revised the article and approved the final draft.

## Conflict of interest and funding

The authors have read and understood the International Committee of Medical Journal Editors (ICMJE) policy on declaration of interests and declare the following interests: within the last 3 years, both EOL and EMHM have received a travel grant from GlaxoSmithKline; RG has received travel grants from the Norwegian Respiratory Society, a grant for the MicroCOPD study from GlaxoSmithKline, and speaker fees from AstraZeneca and Boehringer Ingelheim; TMLE has received travel grants from InterMune for the AIR conferences, a grant for the MicroILD study from Boehringer Ingelheim, and speaker fees from AstraZeneca and Boehringer Ingelheim; and PSB has acted as an advisory board member for Boehringer-Ingelheim, Mundipharma, AstraZeneca. This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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Paper II

BMJ Open Respiratory Research

# Complications and discomfort after research bronchoscopy in the MicroCOPD study

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## ABSTRACT

**Background** Data on discomfort and complications from research bronchoscopy in chronic obstructive pulmonary disease (COPD) and asthma is limited. We present complications and discomfort occurring within a week after bronchoscopy, and investigate personal and procedural risk factors.

**Methods** 239 subjects with COPD, asthma or without lung disease underwent research bronchoscopies as part of a microbiome study of the lower airways (the MicroCOPD study). Bronchoscopy was done in the supine position with oral scope insertion with the option of light conscious alfentanil sedation. Sampling consisted of protected specimen brushes, bronchoalveolar lavage, small volume lavage and for some, endobronchial biopsies. Bleeding, desaturation, cough, haemodynamic changes, dyspnoea and other events that required an unplanned intervention or early termination of bronchoscopy were prospectively recorded. Follow-up consisted of a telephone interview where subjects rated discomfort and answered questions about fever sensation and respiratory symptoms in the week following bronchoscopy.

**Results** An unplanned intervention or early termination of bronchoscopy was required in 25.9% of bronchoscopies. Three subjects (1.3%) experienced potentially severe complications, of which all recovered without sequelae. COPD subjects experienced more dyspnoea than controls. Sedation and lower age was associated with less unplanned intervention or premature termination. About half of the subjects (47.7%) reported fever. Discomfort was associated with postprocedural fever, dread of bronchoscopy, higher score on the COPD Assessment Test and never-smoking. In subjects undergoing more than one bronchoscopy, the first bronchoscopy was often predictive for complications and postprocedural fever in the repeated bronchoscopy.

**Conclusion** Research bronchoscopies were not associated with more complications or discomfort in COPD subjects. 47.7% experienced postbronchoscopy fever sensation, which was associated with discomfort.

## INTRODUCTION

Bronchoscopy is a standard diagnostic procedure in lung cancer and interstitial lung disease. In addition, many patients with obstructive lung disease undergo bronchoscopy as part of differential diagnostics or for microbial sampling.

The reported complication rates of bronchoscopy vary considerably. For instance bleeding varies from 2.5% to 100% of procedures[1 2] and desaturation from 0.7% to 76.3% of procedures.[3 4] Fever, perhaps more of a discomfort than a complication, occurs in 2%–33% of bronchoscopies.[5 6] This variation in reported rates can be attributed to a lack of sufficiently powered studies with clearly defined outcomes, and to a heterogeneity in study populations and local practices. The paucity of information about specific procedure-related and patient-related factors, also applies to bronchoscopy in high-prevalent illnesses such as chronic obstructive pulmonary disease (COPD).[7] Accurate knowledge would serve to better prepare patients and prime bronchoscopists' awareness of possible discomforts and complications for patients undergoing bronchoscopy.

In the Bergen COPD microbiome study (MicroCOPD)[8] we performed more than 300 research bronchoscopies in subjects with and without obstructive pulmonary disease. The current analysis investigates if research bronchoscopy is less safe in subjects with obstructive lung disease by evaluating complications and discomfort occurring immediately, and within a week after bronchoscopy.

## MATERIALS AND METHODS

### Study population

The MicroCOPD study included COPD and asthma patients as well as subjects without lung or airways disease ('controls').[8] Participants were recruited from the Bergen COPD Cohort Study[9] and the GenKOLS Study,[10] in addition to volunteers from the outpatient clinic at the Department of Thoracic

Medicine, Haukeland University Hospital and asthma patients from a local pulmonology clinic. COPD and control subjects were 40 years or older. The COPD and asthma diagnoses were verified by experienced pulmonologists based on spirometry (COPD: postbronchodilation forced expiratory volume in 1 second/forced vital capacity ($FEV_1$/FVC)<0.7, according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines[11]), respiratory symptoms, disease history and other diagnostic modalities such as CT of the lungs. No bronchoprovocation challenge was conducted. Control subjects were subjects that did not have symptoms or lung function tests compatible with a diagnosis of airways disease. A pilot study was conducted with eight COPD subjects before starting the main study, as part of protocol development. All participants provided written informed consent prior to inclusion.

### Selection for bronchoscopy

Participation was postponed in subjects that had been treated for a COPD exacerbation within the last 2 weeks, or who had ongoing respiratory symptoms. Bronchoscopy was not performed in subjects that were hypoxemic despite oxygen supplementation ($O_2$ saturation <90%), hypercapnic, at increased risk of bleeding, had known allergy towards the premedication, or had cardiac risk factors as specified in the protocol.[8]

### Bronchoscopy procedure

Bronchoscopy was performed by one of six bronchoscopists with the subject in the supine position, through oral access and either with or without light conscious sedation according to the subjects' preference, with intravenous alfentanil (0.25–1.0 mg). In addition to salbutamol administration related to the preceding spirometry, asthmatics received 5 mg of nebulised salbutamol and in some cases also 0.5 mg of ipratropium bromide (per judgement of the bronchoscopist). All participants received topical anaesthesia (lidocaine) by oral spray formulation (10 mg/dose) prior to the procedure and through a catheter (20 mg/mL) in the bronchoscope's working channel during bronchoscopy. Additional alfentanil was administered during bronchoscopy, if deemed necessary. All participants received supplemental oxygen by nasal cannula, 3 L/min. The procedure included a general inspection, sampling with protective specimen brushes, bronchoalveolar lavage (BAL) of 50 mL +50 mL if $FEV_1$ >30% of predicted, small volume lavage (20 mL), and in one third of bronchoscopies; endobronchial biopsies. The biopsies, up to six in total, were taken from carinas in the right lower lobe after installation of 5 mL of 0.1% epinephrine. A disposable 1.8 mm cupped biopsy forceps was used. Subjects were monitored by three-lead ECG and pulse oximetry throughout the procedure. After bronchoscopy, the subjects were observed by trained nurses in our outpatient clinic for 2 hours. After discharge, the participants received a direct telephone number to the physician that performed their bronchoscopy in case of illness or worries following the procedure.

### Predictors and outcomes

Information about subject-related explanatory variables was collected prior to bronchoscopy. All subjects were evaluated by the COPD Assessment Test (CAT),[12] utilised as a binary (CAT ≥10) variable. COPD and asthma subjects reported the number of exacerbations in the preceding year. Partial oxygen pressure ($PaO_2$) at rest was measured. All subjects underwent spirometry after inhalation of 0.4 mg salbutamol. Norwegian reference values for $FEV_1$ and FVC were used.[13] Subjects were categorised as ex-smokers, current-smokers or never-smokers. Subjects rated dread of the upcoming bronchoscopy on a scale from 0 to 10, with 0 being 'not at all' and 10 being 'worst imaginable'. The six bronchoscopists were divided into a binary more-or-less experienced variable, based on experience level. The two most experienced bronchoscopists were all certified pulmonologists, senior consultants, and with more than 400 bronchoscopies, whereas the four least experienced failed to fulfil one or more of the above criteria.

Procedure-related explanatory variables included premedication with alfentanil and whether biopsies or BAL was performed.

Complications occurring during the procedure and observation period was recorded (online supplementary appendix 1). The main outcome was complications leading to unplanned intervention or premature termination of the procedure. An unplanned intervention was defined as any intervention that was not part of the prespecified bronchoscopy procedure, and deemed necessary by the bronchoscopist during or immediately after bronchoscopy. All supplementary administration of medications, included increased oxygen delivery, was regarded an unplanned intervention. Outcomes of special interest were observed cough, dyspnoea, decrease in oxygen saturation, haemodynamic changes (eg, pulse/blood pressure) and bleeding. To some degree these events are side-effects of the procedure, rather than complications. So, to be considered a complication, the event had to lead to an unplanned intervention. Examples of unplanned interventions included (but were not limited to) additional topical anaesthesia or sedation in the case of cough, increase in oxygen delivery in the case of desaturation, administration of (additional) epinephrine in the case of bleeding, bronchodilators in the case of dyspnoea, intravenous fluids and/or naloxone in the case of light-headedness or an observed reduction in blood pressure and antiemetics in the case of nausea. Severe complications are in this study limited to situations where a participant received urgent healthcare attendance due to a threat to life or health.

Self-reported events and discomfort were recorded in structured interviews that took place on-site after bronchoscopy, and by telephone 1 week after (online

supplementary appendix 2). Discomfort was graded on a 10-point scale, where 0 represented 'no discomfort' and 10 'worst discomfort imaginable'. Participants were asked about willingness to repeat the procedure, and whether they had experienced fever sensation (temperature was not measured), dyspnoea, sputum, rhinitis, wheezing chest sounds, sore throat, cough, fatigue, haemoptysis and feeling of influenza (muscle/joint ache, fever, headache, malaise). Respiratory symptom exacerbations within the following week were defined according to modified Anthonisen criteria.[14] All healthcare utilisations in the week following bronchoscopy (medication use, exacerbation treatment and hospitalisation) was recorded.

### Repeated bronchoscopies

In a non-random selection, some participants were invited to undergo a repeated second, and in a few cases, third bronchoscopy. For each repeated bronchoscopy procedure, all information on the subject and the bronchoscopy procedure was recorded again.

### Statistics

Bivariate analyses of explanatory and outcome variables in COPD and controls were performed using parametric (t-test, paired t-test) and non-parametric tests ($\chi^2$, Fisher's exact test, Cohen's kappa, quantile regression). For subjects undergoing more than one bronchoscopy, the outcomes of the first and second bronchoscopy were compared. Data from asthma subjects were included in the regression models and in the overall descriptive statistics. However, comparison between asthmatics and the COPD and control groups was not performed due to the low number of asthmatics included. A logistic regression model for the dichotomous combined variable of unplanned intervention and/or premature termination of bronchoscopy and a quantile (median) regression model for the outcome of discomfort were fitted. In the multivariate regression models, age and sex were always included, with additional variables added based on bivariate effect size. Predictors were kept for the final model if $p<0.1$ by a likelihood-ratio-test. Analyses were performed using R V.3.4.3 and V.3.6.1 and Stata V.14 for Windows and Stata V.15 for Mac.

### Patient and public involvement

User involvement in the MicroCOPD study has been represented through informal contacts between our bronchoscopists/nurses and their patients, as well as regular meetings between the Department of Thoracic medicine and patient interest organisations such as The Norwegian Association of Heart and Lung Patients and The Norwegian Asthma and Allergy Association.

## RESULTS

Five bronchoscopies were interrupted before bronchoscopic sampling started, and were excluded from further analyses. In one case, the cause of interruption was unreported. In four of these cases, interruption was due to a choking sensation when accessing the larynx and thereby difficult passage of the scope. Three out of these four subjects had received 0.5 mg alfentanil. The eight participants from the pilot study and two volunteer co-workers were also excluded. The current analyses are thus based on 239 subjects (122 COPD, 16 asthma, 101 controls) undergoing bronchoscopy, of which 61 underwent two bronchoscopies and 11 underwent a third. Study design

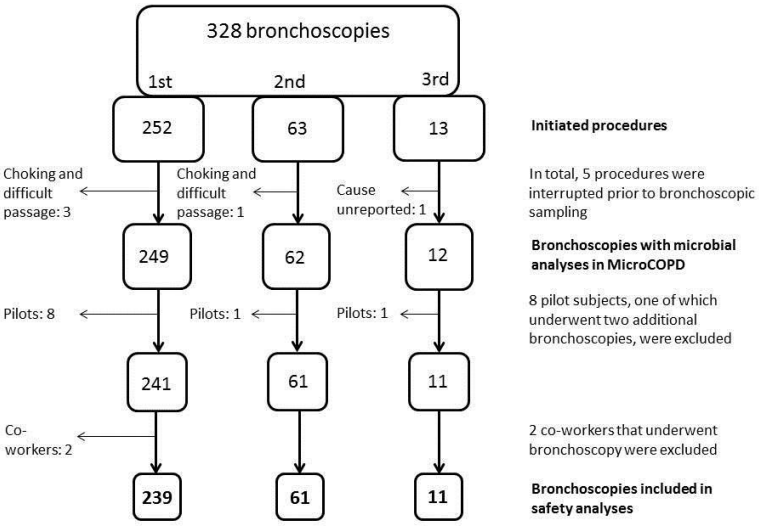


**Figure 1** Study design. COPD, chronic obstructive pulmonary disease; MicroCOPD, Bergen COPD Microbiome Study.

Table 1 Demographic and procedural characteristics in the different study groups

| Variable | COPD n=122 | Asthma n=16 | Control n=101 | Comparison between COPD and control group, two-sided. |
|---|---|---|---|---|
| Female sex | 44.3% | 56.3% | 42.6% | p=0.80 |
| Age, years (SD) | 67.4 (7.3) | 65.5 (12.6) | 65.7 (7.9) | p=0.11 |
| Body mass index (SD) | 26.6 (4.7) | 25.1 (2.9) | 26.7 (3.8) | p=0.81 |
| Smoking status | | | | p≤0.01 |
| Daily | 23.8% | 0.0% | 24.8% | |
| Ex-smokers | 75.4% | 75.0% | 58.4% | |
| Never | 0.8% | 25.0% | 16.8% | |
| $FEV_1$/FVC ratio (SD) | 0.46 (0.13) | 0.67 (0.09) | 0.74 (0.05) | p≤0.01 |
| $FEV_1$ % of predicted (SD) | 56.1 (19.7) | 90.7 (13.3) | 103.9 (12.4) | p≤0.01 |
| GOLD | | | | |
| I | 8.2 % | – | – | |
| II | 50.8 % | – | – | |
| III | 24.6 % | – | – | |
| IV | 16.4 % | – | – | |
| CAT score ≥10 | 79.5% | 68.8% | 26.7% | p≤0.01 |
| $PaO_2$ (SD)* | 9.6 (1.2) | 10.8 (1.1) | 11.1 (1.1) | p≤0.01 |
| $PaCO_2$ (SD)* | 5.2 (0.5) | 4.95 (0.3) | 5.2 (0.5) | p=0.31 |
| Exacerbation ≥2 prev. year† | 17.2% | 6.25% | – | |
| Dread of procedure (SD)‡ | 4.0 (2.8) | 3.5 (2.4) | 3.3 (2.6) | p=0.07 |
| Received alfentanil sedation | 90.2% | 100% | 83.2% | p=0.122 |
| Total lidocaine dose, mg (SD) | 475 (54) | 479 (58) | 458 (45) | p=0.01 |
| BAL performed | 78.7% | 87.5% | 96.0% | p≤0.01 |
| Biopsies performed | 39.3% | 87.5% | 37.6% | p=0.79 |
| Less experienced bronchoscopist | 63.1% | 43.8% | 59.4% | p=0.57 |

Dread of procedure was rated on a 0–10 scale, with 0 representing no dread and 10 worst dread.
*Three missing values (one control, two COPD).
†Five missing values (one COPD, four asthma).
‡20 missing values (11 COPD, eight controls, one asthma).
BAL, bronchoalveolar lavage; CAT, COPD assessment test; COPD, chronic obstructive pulmonary disease; $FEV_1$, forced expiratory volume after 1 second; FVC, forced vital capacity; GOLD, Global Initiative for Chronic Lung Disease stage; $PaCO_2$, partial pressure of carbon dioxide; $PaO_2$, partial pressure of oxygen; prev, previous.

is shown in figure 1. Mean procedure duration was 14.2 min (SD 4.0). Subject and procedure characteristics at baseline are given in table 1.

### First bronchoscopy; observed outcomes

Periprocedural events requiring an unplanned intervention or early termination of bronchoscopy occurred in 25.9% of subjects. The majority of events were minor reactions, like cough, handled by alfentanil or lidocaine administration. No subject received more than 1.0 mg alfentanil in total. Early termination occurred in 15 (6.3%) of the procedures. The most frequent procedural events were cough, desaturation and bleeding (table 2). The seven bleeding events requiring an intervention resolved quickly after epinephrine administration. None required surgical intervention or transfusion.

Noted haemodynamic changes not requiring intervention were mainly elevations in heart rate and blood pressure during bronchoscopy. All but one haemodynamic change that led to intervention were decreases in BP that led to administration of either naloxone or intravenous fluids. The one increase in BP was accompanied by nausea, and antiemetic treatment was given.

Within the 2-hour observation period after bronchoscopy, the most common complications were dyspnoea (n=11) and sedation side effects (light-headedness, nausea) leading to intravenous naloxone or metoclopramide hydrochloride administration (n=10) (table 2). Only COPD subjects experienced dyspnoea requiring bronchodilators. For other observed immediate complications, there was no statistically significant difference between the two groups (table 2).

Three patients had potentially severe complications requiring immediate healthcare attendance: One COPD subject became unconscious 1 hour after the procedure and recovered after naloxone administration. One asthma subject syncopated during the first interview shortly after bronchoscopy, while still being monitored with ECG. At the time of syncopation, the monitor showed a bradycard rhythm, that was perceived as an asystole, and short cardiopulmonary resuscitation was initiated. The subject regained consciousness before respiration and rhythm/pulse was evaluated, and before administration of naloxone. Naloxone was provided shortly after. Both participants that syncopated had received 0.5 mg of alfentanil as premedication. The procedures were uneventful, with no need of additional oxygen or medication. One asthma subject experienced bronchospasm at the end of an otherwise uneventful procedure and was treated with intravenous bronchodilators. The two asthma subjects were hospitalised for 24 hours. All recovered quickly without sequelae (table 2).

There were fewer unplanned interventions and/or premature terminations in subjects receiving alfentanil (OR 0.27, CI 0.11 to 0.66), and more in subjects with higher age (OR 1.73, CI 1.13 to 2.63) (figure 2). Subjects without alfentanil sedation did not receive different amounts of lidocaine during bronchoscopy (p=0.14).

**Table 2** Procedural complications of research bronchoscopy

| | COPD | Asthma | Controls | Comparison, |
|---|---|---|---|---|
| N | 122 | 16 | 101 | COPD/controls |
| Cough during bronchoscopy | | | | p=0.81 |
| Without need for intervention | 18.9% | 12.5% | 14.9% | |
| In need of intervention | 4.1% | 6.3% | 5.9% | |
| Leading to early termination of procedure | 2.5% | 6.3% | 3.0% | |
| Bleeding during bronchoscopy | | | | p=0.06 |
| Without need for intervention | 9.0% | 12.5% | 4.0% | |
| In need of intervention | 3.3% | 12.5% | 0.0% | |
| Leading to early termination of procedure | 0.8% | 0.0% | 0.0% | |
| Desaturation during bronchoscopy | | | | p=0.29 |
| Without need for intervention | 27.1% | 18.8% | 35.6% | |
| In need of intervention | 4.1% | 6.3% | 1.0% | |
| Leading to early termination of procedure | 0.0% | 0.0% | 1.0% | |
| Measurement failure | 2.5% | 0.0% | 2.0% | |
| Haemodynamic changes* | | | | p=0.38 |
| Without need for intervention | 15.6% | 12.5% | 9.9% | |
| In need of intervention | 0.8% | 6.3% | 2.0% | |
| Leading to early termination of procedure | 0.0% | 0.0% | 1.0% | |
| Measurement failure | 0.8% | 0.0% | 0.0% | |
| Retching leading to change of bronchoscope during bronchoscopy | 0.0% | 0.0% | 1.0% | p=0.45 |
| Retching leading to early termination | 0.0% | 0.0% | 3.0% | p=0.09 |
| Panic, subject unease | 1.6% | 0.0% | 0.0% | p=0.50 |
| Total amount of early terminated bronchoscopies, all reasons. | 4.9% | 6.3% | 7.9% | p=0.36 |
| Potentially severe complications immediately after bronchoscopy | | | | |
| Bronchospasm immediately after bronchoscopy | 0.0% | 6.3% | 0.0% | |
| Syncope, rescued by naloxone | 0.8% | 0.0% | 0.0% | |
| Syncope, started resuscitation | 0.0% | 6.3% | 0.0% | |
| Dyspnoea immediately after bronchoscopy | | | | p<0.01 |
| Without need for intervention | 2.5% | 0.0% | 1.0% | |
| In need of intervention | 8.2% | 6.3% | 0.0% | |
| Postprocedural reactions leading to use of metoclopramide hydrochloride and/or naloxone | 3.3% | 6.3% | 5.0% | p=0.74 |
| Requiring any intervention or early termination of bronchoscopy, total | 26.2% | 37.5% | 23.8% | p=0.67 |

*Not including complications listed under 'severe complications'.
COPD, chronic obstructive pulmonary disease.

## First bronchoscopy; self-reported outcomes

Sensation of fever was reported by 47.7% (table 3). There was no difference between those who had BAL performed and those who did not. COPD subjects reported more dyspnoea and increased wheezing sounds than the controls in the week following bronchoscopy. There was no difference between COPD and control subjects regarding other respiratory symptoms or exacerbation criteria (table 3).

Significant predictors of the 10-point discomfort scale were postprocedural fever, dread of bronchoscopy and being a never-smoker (table 4).

Seven COPD subjects (5.7%) received antibiotic treatment or oral corticosteroids in the week following bronchoscopy, compared with one control subject (table 3).

One COPD subject had a suspected transient ischaemic attack 4 days after bronchoscopy. A magnetic resonance scan of the brain showed chronic circulatory disturbances. We classified this event as having an uncertain relation to the bronchoscopy.

Willingness to return for a research bronchoscopy was 79.8%, and was not different between the COPD and control group (table 3). Among subjects unwilling to return, 87.2% would undergo bronchoscopy if recommended by a physician.

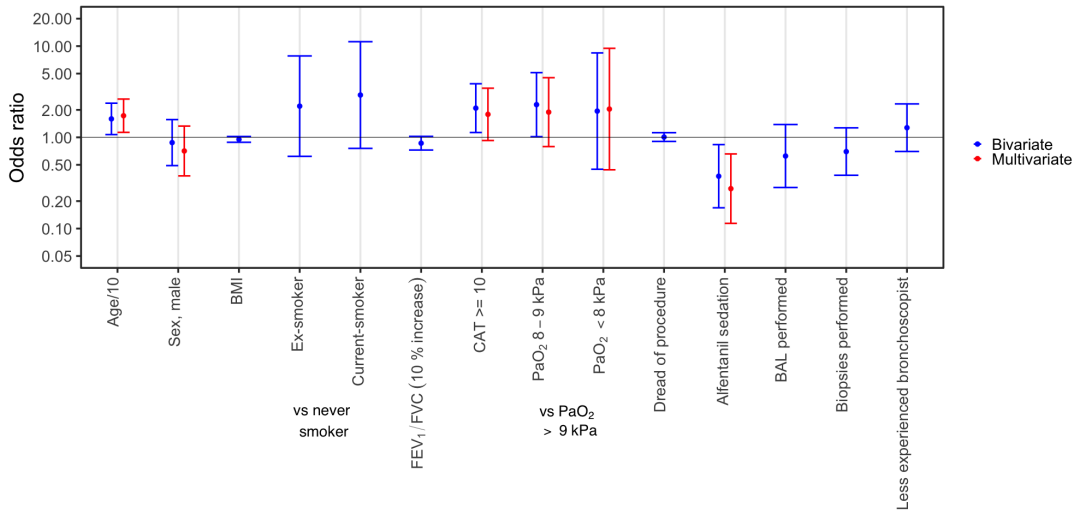


**Figure 2** Logistic regression was used to evaluate the combined outcome of unplanned intervention or premature termination of bronchoscopy. Total number of observations in this model was 236, as three observations were omitted due to missing values of oxygen. BAL, bronchoalveolar lavage; BMI, body mass index; CAT, COPD assessment test; COPD, chronic obstructive pulmonary disease; $FEV_1$, forced expiratory volume in 1 second; FVC, forced vital capacity; $PaO_2$, partial oxygen pressure.

### Second bronchoscopy

Among the 61 subjects who underwent a second bronchoscopy, the total complication rate of the first bronchoscopy was 20%. Of those with a complicated first bronchoscopy, 42% had a complicated second bronchoscopy. In the group with no event in the first

**Table 3** Self-reported outcomes of research bronchoscopy after 1 week

| | COPD n=122 | Asthma n=16 | Controls n=101 | Comparison, COPD/controls |
|---|---|---|---|---|
| Willingness to return for research bronchoscopy* | 76.2% | 68.8% | 84.2% | p=0.213 |
| Fever sensation† | 45.9% | 37.5% | 51.5% | p=0.440 |
| Increased dyspnoea† | 31.4% | 25.0% | 13.9% | p=0.002 |
| Increased sputum† | 26.2% | 25.0% | 22.8% | p=0.540 |
| Change in sputum colour† | 20.7% | 25.0% | 12.9% | p=0.125 |
| Increased rhinitis† | 31.4% | 25.0% | 31.7% | p=0.965 |
| Increased wheezing respiration† | 24.8% | 12.5% | 7.9% | p=0.001 |
| Sore throat or coughing† | 54.1% | 56.3% | 57.4% | p=0.601 |
| Increased asthenia‡ | 37.2% | 31.3% | 27.0% | p=0.108 |
| Flu-like symptoms, including fever, muscle/joint pain, headache, reduced general condition* | 41.7% | 31.3% | 50.5% | p=0.189 |
| Discomfort graded from 0 to 10. Mean (SD)* | 4.2 (2.6) | 3.8 (2.8) | 4.2 (1.9) | p=0.364 |
| Exacerbation criteria fulfilled, total‡ | 45.5% | 37.5% | 33.0% | p=0.169 |
| Hospitalisation related to bronchoscopy§ | 0.8% | 12.5% | 1.0% | p=1.000 |
| Received treatment as if exacerbation (prednisolone/antibiotics)§ | 5.7% | 0.0% | 1.0% | p=0.076 |

*Two missing values (two COPD).
†One missing value (one COPD),
‡Two missing values (one COPD, one control).
§One missing value (one control).
COPD, chronic obstructive pulmonary disease.

bronchoscopy, 12% had a complicated second bronchoscopy (p=0.01).

Especially sensation of fever after the first bronchoscopy was associated with similar reports after a second bronchoscopy (p<0.01). Among subjects undergoing a second bronchoscopy, 45% reported fever after the first bronchoscopy. Of these, 63% experienced fever after the second bronchoscopy. Of those who did not report fever in their first bronchoscopy, only 27% reported fever after the second procedure.

## DISCUSSION

In our single-centre bronchoscopy study we found that only 1.3% of 239 participants experienced serious complications, all of whom had a diagnosis of COPD or asthma. No complication had long-term consequences. Of first bronchoscopies, 6.3% were prematurely terminated. As it can be unclear what constitutes a complication or an expected discomfort, we chose to define a complication as an observed event that led to an unplanned intervention, and we chose to let subjects report overall discomfort during the week after the procedure. The most frequent complications were cough, dyspnoea and other discomforts leading to administration of naloxone or metoclopramide hydrochloride. The most common discomforts reported after 1 week were sore throat, fever and flu-like symptoms.

Although one fourth of the subjects required some form of unplanned intervention, it is important to point out that our definition of unplanned intervention was made quite wide to capture as many events as may be of any significance. However, many events will regularly happen during a routine bronchoscopy, like cough or light bleeding, being routinely handled by extra

**Table 4** Predictors of perceived discomfort during and after bronchoscopy, estimated from a quantile regression analysis

| Variable | Coef. | CIs | | Type* | P value |
|---|---|---|---|---|---|
| | | Lower | Upper | | |
| Sex, male | −1 | −1.91 | −0.08 | Bivariate | 0.03 |
| Age/10 | −0.60 | −1.11 | −0.09 | Bivariate | 0.22 |
| Body mass index | 0 | −0.10 | 0.10 | Bivariate | 1.00 |
| Smoking status | | | | | |
| Ex-smoker | 0 | −2.06 | 2.06 | Bivariate | 1.00 |
| Current smoker | 0 | −2.37 | 2.37 | Bivariate | 1.00 |
| $FEV_1$/FVC | 0 | −0.03 | 0.03 | Bivariate | 1.00 |
| CAT score ≥10 | 1.5 | 0.48 | 2.52 | Bivariate | <0.01 |
| $PaO_2$ | | | | | |
| $PaO_2$ 8–9 kPa | 0 | −1.79 | 1.79 | Bivariate | 1.00 |
| $PaO_2$ <8 kPa | 1 | −3.42 | 5.42 | Bivariate | 0.66 |
| Dread of procedure | 0.34 | 0.16 | 0.50 | Bivariate | <0.01 |
| Alfentanil sedation | 0 | −1.28 | 1.28 | Bivariate | 1.00 |
| BAL performed | 0 | −1.74 | 1.74 | Bivariate | 1.00 |
| Biopsies performed | 0 | −0.91 | 0.91 | Bivariate | 1.00 |
| Less experienced bronchoscopist | 1 | −0.09 | 2.09 | Bivariate | 0.07 |
| Fever sensation | 1.5 | 0.28 | 2.72 | Bivariate | 0.02 |
| Complication | 0.5 | 0.83 | 1.83 | Bivariate | 0.46 |
| Sex, male | −0.08 | −0.88 | 0.73 | Multivariate | 0.83 |
| Age/10 | −0.36 | −0.84 | 0.11 | Multivariate | 0.14 |
| CAT score ≥10 | 0.62 | −0.17 | 1.41 | Multivariate | 0.12 |
| Fever | 0.87 | 0.09 | 1.65 | Multivariate | 0.03 |
| Dread of procedure | 0.30 | 0.16 | 0.44 | Multivariate | <0.01 |
| Smoking status | | | | | |
| Ex-smoker | −1.35 | −2.34 | −0.35 | Multivariate | 0.01 |
| Current smoker | −2.05 | −3.38 | −0.67 | Multivariate | <0.01 |

Discomfort was rated on a 0–10 scale, with 0 representing no discomfort and 10 worst imaginable discomfort.
*In the multivariate model, age and sex were included and additional variables were added based on bivariate effect size. Predictors were kept for the final model if p<0.1 by a likelihood-ratio-test.
BAL, bronchoalveolar lavage; CAT, COPD assessment test; Coef, Coefficient; $FEV_1$, forced expiratory volume after 1 second; FVC, forced vital capacity; $PaO_2$, partial pressure of oxygen.

medication without any harm to the person undergoing the procedure.

The only statistically significant difference between COPD subjects and controls was more postprocedural dyspnoea in COPD subjects. These findings are in accordance with a recent study that reported similar safety profiles in patients with and without COPD,[15] and previous findings of more respiratory complications in COPD patients following bronchoscopy.[16] Predictors of unplanned intervention or early procedure termination were lack of sedation and higher age. Predictors of reported discomfort were smoking habits, dread of bronchoscopy and postprocedural fever, but effect sizes were small. The reason why ever smokers reported less discomfort is unknown, but one possibility is a higher tolerance for respiratory symptoms.

Aside from more dyspnoea, bronchoscopy of COPD patients was not associated with more complications, even when $FEV_1$ was below 30% of predicted. However, we did not perform BAL in subjects with the most reduced $FEV_1$. Less dyspnoea in the asthma group than in the COPD group could be explained by a low number of asthma patients, but also by preprocedural bronchodilation. The British Thoracic Society guideline for diagnostic flexible bronchoscopy in adults states that nebulised bronchodilators should be considered before bronchoscopy in patients with asthma,[17] whereas no benefit of inhaled salbutamol has been identified in COPD patients.[18]

The low number of asthma subjects makes it impossible to draw conclusions based on complication rates in the asthma group. That the two subjects who were hospitalised directly following bronchoscopy both had asthma could indicate that subjects with asthma are more prone to complications.

Alfentanil reduced overall need for unplanned intervention or early termination of bronchoscopy, even though ten cases of drug-induced complications were included in the analyses. There is no commonly accepted best practice regarding choice of sedative agent for bronchoscopy.[19] Bronchoscopy sedation with alfentanil has only recently been compared with placebo or dexmedetomidine in a relatively small randomised controlled trial. The authors reported more events of hypoxaemia and heart rate changes in the alfentanil group, but present few clinically significant differences between the groups.[20] Older studies comparing alfentanil and midazolam sedation have shown that alfentanil sedation results in less cough, but not necessarily less discomfort or improved ease of the procedure.[21 22] The trial comparing alfentanil to placebo did not find a statistically significant difference in cough, and did not address subject discomfort.[20] Sedation was not randomised in our study, but offered to all. We were unable to find differences in reported discomfort in subjects with and without sedation. A non-recorded observation was that some participants declined sedation to be able to drive a motor vehicle after bronchoscopy. Midazolam is not routinely used at our institution, although sometimes preferred in patients with manifest anxiety for instance. Patients receiving midazolam may relax more, which may have preferable effects, however we cannot assess what impact midazolam may have on procedural discomfort from the current study.

Thus, alfentanil appears to provide clear benefits for the majority of recipients, but does come with the risk of serious events, especially related to depression of respiration. Using a standardised sedation protocol and having an experienced team performing the bronchoscopies is likely beneficial in maximising benefit and minimising risk.

The postbronchoscopy fever rate was in accordance with a paediatric study.[23] Others have reported lower rates (0%–38%).[24–30] The causes of this wide range in rates are unclear, but the studies vary greatly in patient population and design. A possible reason for our relatively high fever rate is that we asked for a self-reported fever, which also included a 'fever sensation' instead of measuring body temperature. The abovementioned paediatric study found more fever in cases where BAL had been performed. In the current study, fever was not associated with BAL. However, BAL was not done in a randomised manner, and was not performed in patients with very severe obstruction.

Postbronchoscopy fever itself is harmless, but is associated with subject discomfort. COPD patients may interpret fever sensation as an early sign of exacerbations. Although subjects reported relatively high scores of discomfort and many had airway exacerbation symptoms, 79.8% would undergo research bronchoscopy again. Our findings on willingness to return fall within the range of previously reported rates,[15 31–34] but might be influenced by response bias.

Of participants undergoing a second bronchoscopy, the first bronchoscopy appears to be predictive for both immediate complications and fever. This indicates that the fever is at least partially subject-related and not procedural-related. To the best of our knowledge, there are no other studies on the complication or discomfort rate in repeated bronchoscopies.

Our analyses suggest that exacerbation symptoms may increase after bronchoscopy. Thus, patients might profit from an increase in their bronchodilators or inhaled steroids before and after the procedure. Dread of bronchoscopy before the procedure predicted discomfort, suggesting an anxiety relieving effect of information. Knowledge about factors influencing discomfort may help bronchoscopists improve their preprocedural information.

There are reasons why caution should be used when comparing our results to the clinical bronchoscopy setting. First, the patients undergoing bronchoscopy may be healthier than patients undergoing bronchoscopy for a clinical indication. Despite setting no upper age-limit (the oldest individual was 82 years old) and including COPD GOLD stage 4, the frailest subjects were excluded. Second, clinical bronchoscopies often have longer

durations and include more invasive techniques than those applied and accepted in our research setting. Diagnostic bronchoscopies investigating potential malignant disease obtains larger and often transbronchial biopsies. Therapeutic bronchoscopies, such as endobronchial coil therapy, is associated with more severe complications (eg, pneumothorax and death).[35] Third, volunteers are possibly more positive towards the procedure than patients in a clinical setting. This could cause them to have an overall more positive experience, resulting in less perceived discomfort. However, they could also report more complications as a result of a greater discrepancy between what they expected and the actual procedure. Fourth, the procedure was standardised. Hence, the bronchoscopist had to make fewer decisions during bronchoscopy, which may lower complication rates of research bronchoscopy.

This descriptive study has some limitations. For many of our descriptive and outcome variables, we used self-reported outcomes. Recording of complications such as dyspnoea, cough, or bleeding will by nature be subjective. Therefore, we reported events that had a consequence in the form of intervention or termination. The perceived need to intervene is however also subjective and at the discretion of each bronchoscopist. Importantly, we did not observe differences in complication or discomfort rates between the bronchoscopists in our study. All subjects were monitored with ECG, pulse oximeter and blood pressure measurements, however these parameters were only noted in the case of an observed event. If these measurements had been systematically collected, they could have been implemented in quality control or further analyses. This could have improved the study and potentially aided in defining cutoffs for future investigations.

## CONCLUSION

Only 1.3% of subjects had a potentially serious complication, all of whom had no sequela, indicating that bronchoscopy applying invasive techniques such as BAL and mucosal biopsies is a safe procedure in studies of patients with obstructive lung disease. Overall, a sizeable number of subjects perceived some discomfort or less serious complications, but these were minor and to a large degree to be expected from the procedure. Sore throat, fever and flu-like symptoms each occurred in roughly half of all subjects. Non-sedation and higher age were significantly associated with more unplanned interventions during bronchoscopy, indicating that sedation improves tolerability of the procedure and is advised. Information regarding expected discomfort should be given prior to bronchoscopy.

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**Contributors** EOL had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. EOL, TMLE, EMHM, EN, GRH, KSK, SL, ØS and RN participated in different aspects of data collection. TMLE, GRH, KSK, SL, ØS and RN performed the bronchoscopies. EOL, TMLE, EMHM, EN, GRH, KSK, SL, ØS, PSB and RN contributed substantially to the study design, data analysis and interpretation, and the writing of the manuscript.

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**Ethics approval** The Regional Committee for Medical and Health Research Ethics approved the project (REK VEST 2011/1307). All participants provided written informed consent.

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**Data availability statement** The data and command lines necessary for running the analyses presented in this article are available upon reasonable request.



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Paper III

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ORIGINAL ARTICLE
COPD

# The airway microbiota and exacerbations of COPD

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ABSTRACT

**Aim:** The aim of this study was to investigate whether the compositionality of the lower airway microbiota predicts later exacerbation risk in persons with COPD in a cohort study.

**Materials and methods:** We collected lower airways microbiota samples by bronchoalveolar lavage and protected specimen brushes, and oral wash samples from 122 participants with COPD. Bacterial DNA was extracted from all samples, before we sequenced the V3-V4 region of the 16S RNA gene. The frequency of moderate and severe COPD exacerbations was surveyed in telephone interviews and in a follow-up visit. Compositional taxonomy and α and β diversity were compared between participants with and without later exacerbations.

**Results:** The four most abundant phyla were Firmicutes, Bacteroidetes, Proteobacteria and Fusobacteria in both groups, and the four most abundant genera were *Streptococcus*, *Veillonella*, *Prevotella* and *Gemella*. The relative abundances of different taxa showed a large variation between samples and individuals, and no statistically significant difference of either compositional taxonomy, or α or β diversity could be found between participants with and without COPD exacerbations within follow-up.

**Conclusion:** The findings from the current study indicate that individual differences in the lower airway microbiota in persons with COPD far outweigh group differences between frequent and nonfrequent COPD exacerbators, and that the compositionality of the microbiota is so complex as to present large challenges for use as a biomarker of later exacerbations.

@ERSpublications
**Contrary to previous reports, in this study there were no significant associations between the lung microbiota in stable COPD and COPD exacerbation frequency** https://bit.ly/2ZVcNdG

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The sequencing data and relevant metadata are available at the DRYAD data repository from https://doi.org/10.5061/dryad.tqjq2bvw3

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## Introduction

COPD exacerbations are often caused by bacterial and viral respiratory infections with common pathogens such as *Haemophilus influenzae, Streptococcus pneumoniae* and *Moraxella catarrhalis* [1]. Before next-generation sequencing was made available, these microbes were detected by growth in cultures and later PCR. PCR studies suggested the aetiology could be multi-microbial, with frequent detection of multiple bacteria and/or viruses [2–4]. However, not all COPD exacerbations show clinical or microbial signs of infection. Exacerbation-associated bacteria have been cultured from sputum obtained in clinically stable COPD, giving rise to a still unresolved debate on whether colonisation is a pathogenic factor for later exacerbation [5]. The finding of a diverse lung microbiome has led to the hypothesis that exacerbations are caused by a dysbiosis in the airway microbiome, and that differences in baseline microbiota might help explain the difference between the frequent and infrequent exacerbator type [6].

Some previous studies comparing sputum microbiota between stable and exacerbated state in COPD indicate there are differences between disease states, but the findings are inconsistent [7–12]. And, two previous retrospective studies indicated that the sputum microbiota in the stable state was different in patients prone to exacerbations compared to patients who do not exacerbate [13, 14]. However, sputum is invariably fraught by contamination of microbes from the upper airways, which is both difficult to adjust for and potentially interferes with our interpretations of the lower airway microbiota.

To address the question of whether the stable-state lower airway microbiota is predictive of later COPD exacerbation, a study should include both COPD patients known to exacerbate and not, sample the lower airways directly, and include a prospective longitudinal follow-up of exacerbation events. The current study is to our knowledge the first such study with this methodology.

## Methods

### *Study population*

COPD participants in the Bergen COPD Microbiome (MicroCOPD) study were recruited among previous participants in the Bergen COPD Cohort Study [15] and the GenKOLS Study [16], in addition to volunteers from the outpatient clinic at the Department of Thoracic Medicine, Haukeland University Hospital. The protocol for the MicroCOPD study is previously published [17].

To be included, all participants had to be >40 years of age. In the current study sample the youngest participant was 49.5 years upon inclusion. The COPD diagnosis was verified by experienced pulmonologists based on spirometry, respiratory symptoms and disease history. Participation was postponed in individuals that had been treated for a respiratory exacerbation within the last 2 weeks or if they had ongoing symptoms of exacerbation according to modified Anthonisen criteria [18]. We excluded individuals deemed not fit for bronchoscopy, namely those who had an $O_2$ saturation <90% despite oxygen supplementation, $CO_2$ tension >6.65 kPa in arterial blood gas analyses, increased risk of bleeding, known allergy towards the premedication or cardiac risk factors as specified in the protocol [17]. Participants were asked about their current medication, smoking history, number of exacerbations treated with systemic steroids or antibiotics and exacerbation-related hospital admissions in the preceding year. All participants underwent spirometry after inhalation of 0.4 mg salbutamol, followed by bronchoscopy. In the MicroCOPD study, a total of 130 participants with COPD were included. For the current analyses, only the 122 participants from the post-pilot phase of the MicroCOPD study were included.

The MicroCOPD study was approved by the regional ethical committee (REK Vest case number 2011/1307). All participants provided oral and written consent.

### *Microbial sampling*

Prior to bronchoscopy, all participants were asked to gargle 10 mL of PBS for oral wash (OW) sampling. Bronchoscopy was performed through oral access. Topical anaesthesia (lidocaine) was delivered by oral spray formulation prior to the procedure and per-operatively through a catheter within the bronchoscope's working channel. Three protective specimen brushes were collected in the right lower lobe (rPSB). The three brushes were cut off using sterile scissors and placed in an Eppendorf tube with 1 mL of PBS. Protected bronchoalveolar lavage (BAL) was performed in all participants with $FEV_1$>30% of predicted: two fractions each containing 50 mL of PBS were installed through a Combicath sterile catheter with a sealed tip, in the right middle lobe and aspirated through the same sterile catheter. The median retrieval was 33 mL in the group that had no exacerbations and 26 mL in the group that had exacerbations ($p=0.04$, Mann–Whitney U-test). For the current study we used the second fraction of BAL (BAL2), which usually had the highest yield. All samples from one participant (OW, BAL2, rPSB) used the same bottle of PBS, and for each participant we analysed one negative control sample from this bottle. A more detailed explanation of handling of negative control samples is presented in the supplementary text.

### *DNA extraction and 16S sequencing*

The detailed protocol for laboratory processing is published [19]. Briefly, bacterial DNA was extracted by enzymatic lysis followed by processing though the FastDNA Spin Kit. Sequencing of the V3-V4 region of the 16S rRNA gene was carried out according to the Illumina 16S Metagenomic Sequencing Library Preparation guide. The V3-V4 region was PCR amplified using 45 cycles and prepared for a subsequent 8-cycle index PCR step using specific primers. The samples were pooled and diluted before 2×300 cycles of paired-end sequencing was performed using reagents from the MiSeq reagent kit v3 (Illumina Inc., San Diego, CA, USA).

### *Exacerbation follow-up*

We collected information on health-seeking behaviour, use of antibiotics and oral steroids in structured, quarterly telephone interviews for 12 months after the bronchoscopy. All participants were also offered a follow-up examination 1–1.5 years after inclusion where we also collected exacerbation history. Exacerbations were self-reported, but only counted if the exacerbation led to any administration of antibiotics and/or oral corticosteroids or hospitalisations (moderate-to-severe exacerbations). Dates reported in the telephone interviews and the physical follow-up examination were used to confirm that the event had not already been recorded and to separate one exacerbation from another. When information on hospitalisation was contradicting, the reason for admittance was unclear (for instance hospital admittance without administration of antibiotics), or the time point for an exacerbation leading to hospitalisation was unknown, the digital hospital records were consulted.

### *Bioinformatic analyses*

Bioinformatic analyses were performed with QIIME 2 [20] (versions 2018.8, 2018.11, 2019.4, 2019.7, 2020.2) and R (versions 3.5.0 and 3.6.0). Sequencing data from the entire MicroCOPD study (including samples not included in the current analyses) were imported batched by Illumina runs into QIIME 2 (version 2018.8) from Casava 1.8 paired-end demultiplexed fastq format. Denoising was performed using the Divisive Amplicon Denoising Algorithm version 2 (DADA2) software package [21] (*via* q2-dada2), and sequences were further processed with VSEARCH [22] (*via* q2-vsearch) for additional chimera removal and then merged to one amplicon sequence variant (ASV) table (QIIME 2, version 2018.8).

All ASVs representing <0.005% of reads (fewer than 3000 reads) were removed, as they were deemed likely to represent sequencing noise [23]. The ASV table was exported from QIIME 2 into R for bioinformatic identification and removal of contaminants with the package Decontam [24], where we used the prevalence-based method with a threshold of 0.3. A more detailed explanation of the data curation and handling of contaminants are given in the supplementary text.

TABLE 1 Participant characteristics at baseline by exacerbation status during follow-up

| Variable | Exacerbation group | No exacerbation group | Exacerbation unknown | Comparison |
|---|---|---|---|---|
| **Subjects n** | 46 | 59 | 17 | NS |
| **Female** | 45.7% | 47.5% | 29.4% | NS |
| **Age years** | 67.2±7.2 | 68.5±7.3 | 64.3±7.4 | NS |
| **Smoking status** | | | | NS |
| Daily | 19.6% | 27.1% | 23.5% | |
| Ex-smoker | 78.3% | 72.9% | 76.5% | |
| Never | 2.2% | 0% | 0% | |
| **$FEV_1$ % pred** | 46.1±17.0 | 60.4±17.3 | 68.3±22.6 | p<0.01 |
| **$FEV_1$/FVC** | 0.40±0.12 | 0.49±0.11 | 0.45±0.12 | p<0.01 |
| **CAT-score**[#] | 19.4±8.1 | 15.5±7.5 | 12.5±6.4 | p=0.01 |
| **$P_{aO_2}$ kPa**[¶] | 9.4±1.2 | 9.8±1.2 | 9.6±1.2 | NS |
| **Exacerbation ⩾2 previous year** | 32.6% | 10.2% | 5.9% | p<0.01 |
| **Daily use of inhaled corticosteroids** | 69.6% | 39.0% | 35.3% | p⩽0.01 |
| **Daily use of oral steroids** | 6.5% | 3.4% | 5.9% | NS |

Data are presented as % or mean±SD, unless otherwise stated. The comparisons were between the exacerbation and no exacerbation groups only, and were tested using t-tests and Chi-squared tests. NS: not significant; $FEV_1$: forced expiratory volume in 1 s; FVC: forced vital capacity; CAT: COPD Assessment Test; $P_{aO_2}$: arterial oxygen tension. [#]: Two missing values; [¶]: three missing values.

The ASV table was then filtered to contain only samples included in the present study. Taxonomy was assigned using a classifier trained on the Human Oral Microbiome Database [25]. All ASVs that were unassigned to phylum level, were checked with the NCBI BLAST tool [26], and all nonbacterial ASVs were removed. Remaining ASVs were aligned with mafft (*via* q2-phylogeny) and a phylogenetic tree was constructed with fasttree2 [27] (*via* q2-phylogeny). Sequences were rarefied at a sampling depth of 1000 prior to α (within sample) and β (between samples) diversity analyses to ensure equal sampling depths (*via* q2-diversity).

### *Statistics*

Differentially abundant features (*i.e.* genera and ASVs) and differences in compositionality were analysed using Analysis of Composition of Microbes (ANCOM) [28] (*via* q2-composition), balance trees [29] (*via* q2-gneiss), ANOVA-like differential expression analysis 2 (ALDEx2) [30] (*via* q2-aldex2) and differential distribution analysis [31] (with R package MicrobiomeDDA). The α diversity difference, measured with the Shannon index and Faith phylogenetic diversity, was tested with the Kruskal–Wallis test. The β diversity difference, measured with Bray–Curtis and weighted UniFrac distance, was tested with the ADONIS permutation-based test using the vegan package in R [32].

## Results

Of the 122 participants, 105 had complete follow-up of exacerbations for a full year after bronchoscopy. Participants who experienced one or more exacerbations within follow-up had significantly lower lung function, higher symptom score, more frequent exacerbations in the preceding year and more use of inhaled corticosteroids (ICSs) (table 1). Median time to first exacerbation was 146 days. Exacerbations were evenly distributed across seasons of the year.

For the current analyses, we selected the 327 samples of BAL2, OW and rPSB from the 122 participants, of which three samples were judged as only containing nonbacterial taxa and filtered out. In addition, we removed the samples from the 17 participants with unknown exacerbation status. For diversity analyses, rarefied data included 235 samples. Figure 1 shows the flow chart for the sample processing.

### *Taxonomy and differential abundance testing*

Taxonomy at the phylum and genus levels for the three sample types by exacerbation status are shown in figures 2 and 3. Overall, the most abundant phyla were Firmicutes, Bacteroidetes, Proteobacteria and Fusobacteria. The most abundant genera were *Streptococcus*, *Veillonella*, *Prevotella* and *Gemella*. However, the relative abundances of different taxa showed a large variation between samples. Four participants with one or more later exacerbations and one participant without later exacerbations presented with high abundances of the *Moraxella* genus in rPSB samples, which was also reflected by detection of *Moraxella* in BAL2 for those participants who had a lavage sample. When we sorted rPSB samples by relative abundance of the most common taxon, the visual pattern differed from that in BAL2 and OW (figures 2 and 3).

The visual impression from both figure 2 (phylum level) and figure 3 (genus level) was that participants without later exacerbations had more Firmicutes (figure 2), and more streptococci (figure 3), than participants with later exacerbations. However, when testing for statistical significance in difference of abundance between the two groups, few were found. ANCOM, balance trees (gneiss) and ALDEx2 performed on BAL2 and rPSB did not identify any differentially abundant ASVs or genera between the group with and without exacerbations. Differential distribution analysis identified two ASVs as differentially expressed between the groups in rPSB samples (supplementary table 1). These two ASVs were classified as *Capnocytophaga gingivitis* (more abundant, less prevalent and less dispersed in group with exacerbations (adjusted p=0.011)) and *Prevotella pallens* (less abundant, more prevalent and more dispersed in group with exacerbations (adjusted p=0.041)).

To see whether patients who had two or more exacerbations during follow-up differed from those who had zero or one, we repeated the above analyses on this outcome (supplementary table 2). Again, ANCOM, balance trees (gneiss) and ALDEx2 performed on BAL2 and rPSB did not identify any differentially abundant ASVs or genera between the groups. However, differential distribution analysis identified two ASVs (*Capnocytophaga leadbetteri*, adjusted p=0.008, and *Prevotella oris*, adjusted p=0.045) and one genus (*Moraxella*, adjusted p=0.026) as differentially expressed between the groups in BAL samples.

Next, we checked whether the taxonomy by sample type and exacerbation status varied by use of ICSs. The bacterial composition did not appear altered by ICS use, as illustrated in a heat map of genera in BAL2 (figure 4). This impression was strengthened by differential abundance testing by the same four tests

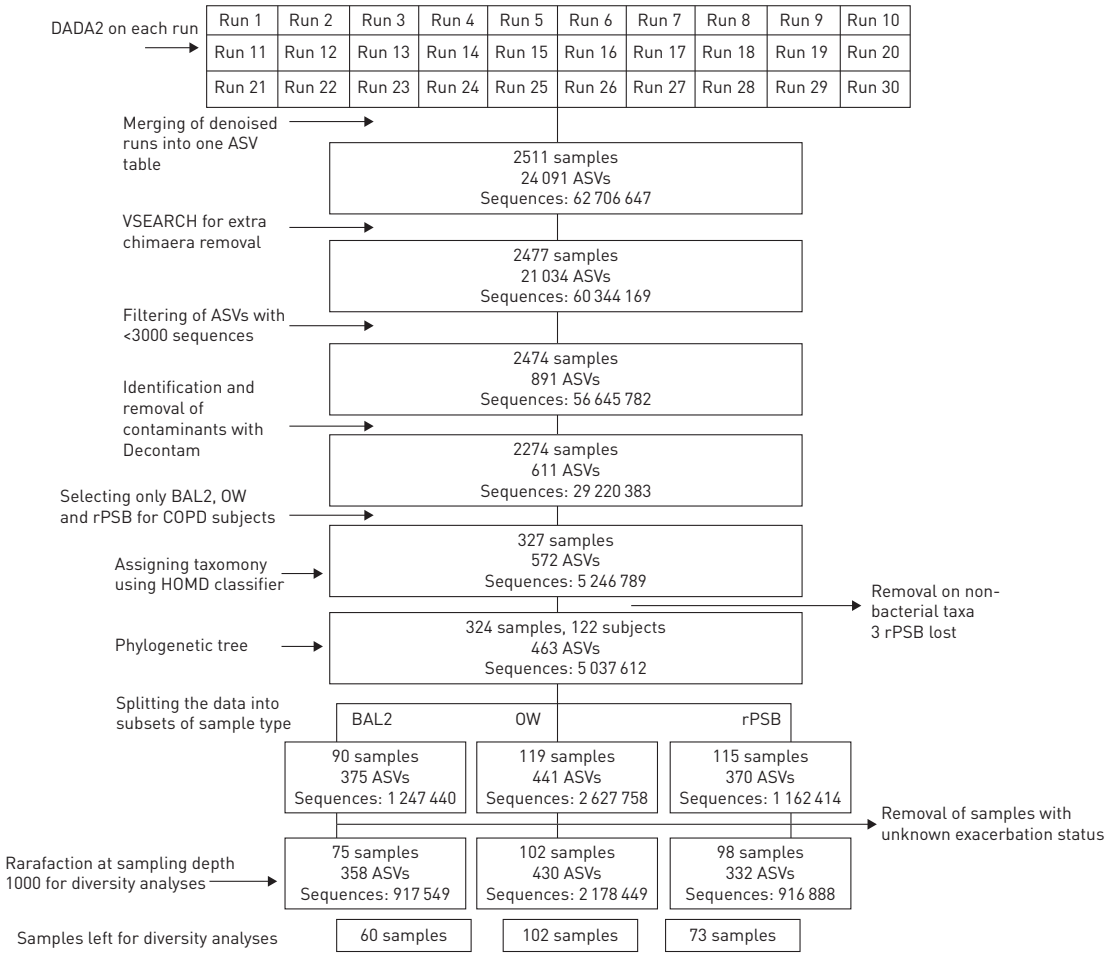


FIGURE 1 Sample flow chart of data processing. DADA2: Divisive Amplicon Denoising Algorithm 2; ASV: amplicon sequence variant; BAL2: second fraction of bronchoalveolar lavage; OW: oral wash; rPSB: protected specimen brushes from the right lung; HOMD: Human oral microbiome database.

as above, revealing no significant differences in taxa between COPD patients who used ICSs and COPD patients who did not (supplementary table 3).

Finally, we examined whether taxonomy differed by previous exacerbations the last year before bronchoscopy (supplemental table 4). Again, only the differential distribution analysis yielded a potential difference, with *Actinomyces graevenitzii* being less abundant and more prevalent in patients having previous exacerbations (adjusted p=0.011).

Overall the main message from the differential abundance tests was that very few differences were found.

### *Diversity*

The α diversity in BAL 2 and rPSB, measured by Faith phylogenetic diversity and Shannon diversity index, did not significantly differ between the group with and without later exacerbations. Box plots of the α diversity are presented in the supplementary material (supplementary figures 5 and 6).

Neither β diversity, measured by Bray–Curtis distance (nonphylogenetic) and weighted UniFrac distance (phylogenetic), differed by exacerbation status (supplementary figures 7 and 8). The four rPSB samples

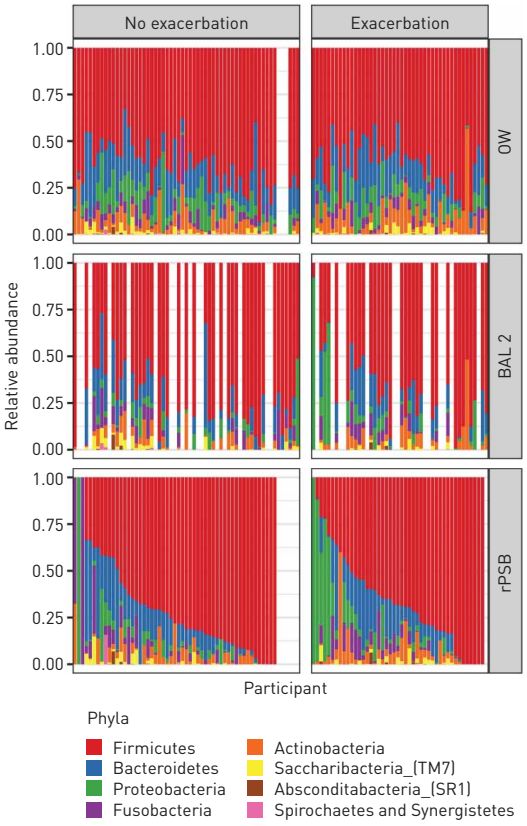


FIGURE 2 Bacterial taxonomy at the phylum level in participants with COPD with and without exacerbation during follow-up. Taxonomic groups in the legend are sorted in decreasing order based on the average relative frequency of that group in all samples. Each bar represents one participant, ordered in the same position horizontally for all three sample types according to relative abundance of Firmicutes in rPSB samples. OW: oral wash; BAL2: second fraction of bronchoalveolar lavage; rPSB: right protected specimen brushes.

and three BAL2 samples that clustered separately (based on weighted UniFrac distance, supplementary figure 9) were the samples with high abundances of *Moraxella* (figure 3). Both unadjusted and adjusted (age, sex, $FEV_1$, ICS use) PERMANOVA analyses showed nonsignificant differences in weighted UniFrac distances between patients with and without later exacerbations (supplementary tables 5 and 6 and supplementary figure 9).

## Discussion

This study did not show any statistically significant characteristics of the lung microbiota in stable COPD, neither in differential abundance of taxonomy, nor α or β diversity, that could predict whether the participants would experience moderate or severe exacerbations in the subsequent follow-up. These findings challenge the few existing previous reports.

Pragman *et al.* [14] reported lower α diversity in sputum from patients with frequent exacerbations, and a significant difference in ASV counts between frequent (n=11) and infrequent (n=11) exacerbators, where *Actinomyces* was found more abundant in patients with infrequent exacerbations. In another recent study, there was an increased abundance of *Pseudomonas*, *Selenomonas* and *Anaerococcus*, in sputum from patients with frequent exacerbations (n=31) compared with patients without exacerbations (n=23). There was also a significant difference in β diversity between patients with frequent and infrequent exacerbations [13]. A study of the microbiota in BAL from patients with stable COPD retrospectively recorded the exacerbation history of 21 patients and suggested that *Streptococcus* and *Rothia* species might be protective and that *Pseudomonas* might be predictive of exacerbations [33].

The current study has superior methodology with a 2–5 times larger sample size than previous studies, prospective follow-up of exacerbations and protected sampling of the lower airways. However, other potential causes of the differences in results between our study and previous studies are worthy of

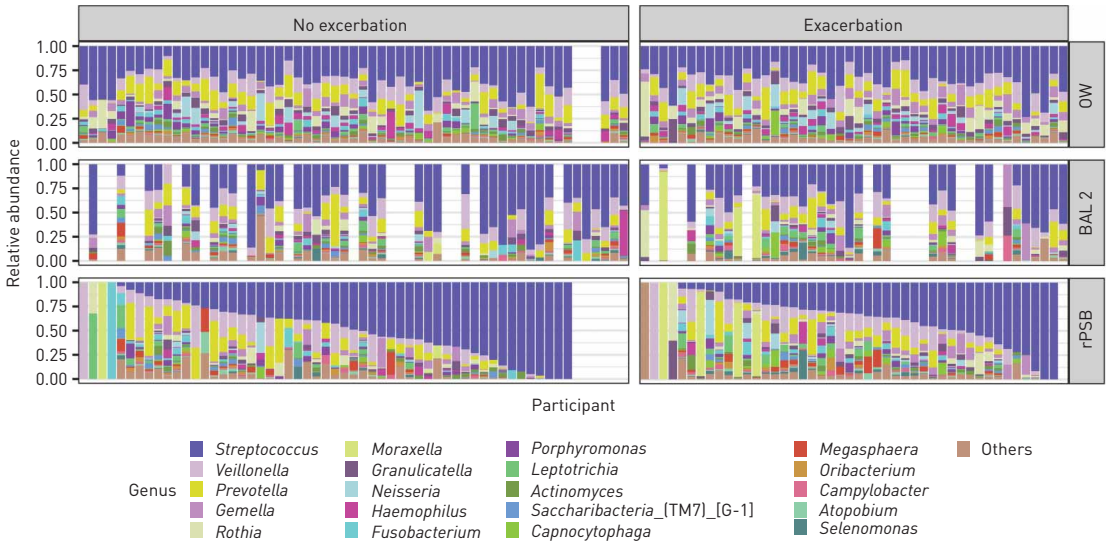


FIGURE 3 Bacterial taxonomy at the genus level in participants with COPD with and without exacerbation during follow-up. Taxonomic groups in the legend are sorted in decreasing order based on the average relative frequency of that group in all samples. Each bar represents one participant, ordered in the same position horizontally for all three sample types according to relative abundance of *Streptococcus* in rPSB samples. OW: oral wash; BAL2: second fraction of bronchoalveolar lavage; rPSB: right protected specimen brushes.

consideration. Two of the previous studies suggest that *Pseudomonas* is associated with more frequent exacerbations, whereas we did not report *Pseudomonas* in any of our samples. Some *Pseudomonas* species are known colonisers in patients with severe disease or impaired immunity, but *Pseudomonas* is ubiquitous in the environment, and there is a high potential for *Pseudomonas* to be a contaminant. The differences between our study and the two previous reports could reflect differences between study populations, but is more likely caused by *Pseudomonas* being identified as a contaminant by the Decontam algorithm in our study, and therefore removed. In the filtered data, *Pseudomonas* was completely removed. In unfiltered

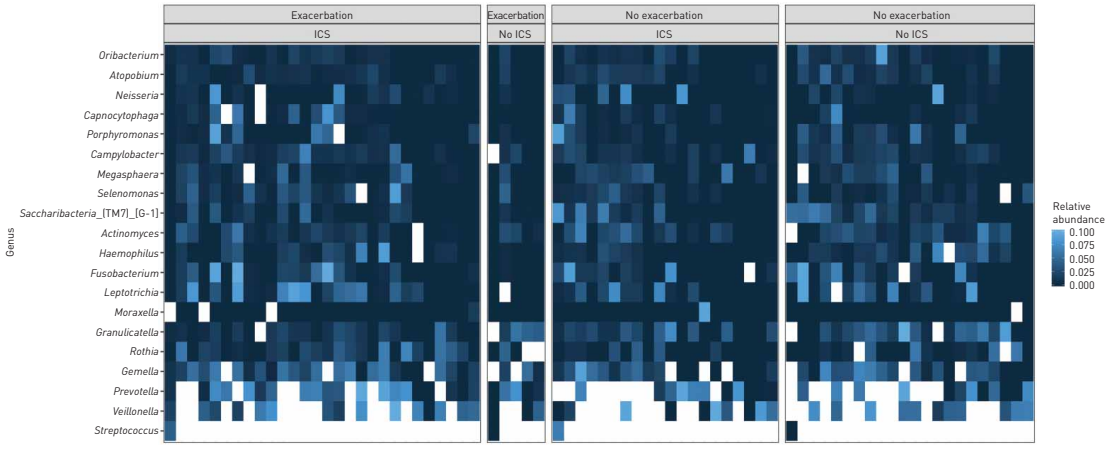


FIGURE 4 Taxonomy in bronchoalveolar lavage. Heat map of 20 most common taxa at genus level. Divided into exacerbation and inhaled corticosteroid categories. All features with a relative abundance above 10% are adjusted white in order to better visualise the less common features. Within each category, samples are ordered according to increasing abundance of *Streptococcus*.

data, *Pseudomonas* was identified in high abundances in samples from participants both with and without later exacerbations.

The overall abundance of *Streptococcus* in our study appeared by visual inspection higher in those participants without later exacerbation (figures 2 and 3), perhaps supporting the suggestion of this taxa being protective as suggested by Ren *et al.* [33]. However, none of the four differential abundance tests we performed found *Streptococcus* to be differentially abundant. The compositionality of data, the lack of quantitative measures, and the low taxonomic resolution of 16S sequencing for *Streptococcus* are challenges that may explain why no association was detected, in addition to the obvious possibility that no association actually exists. We also found that the differences in abundance within groups were bigger than between groups. To date, there is no consensus on what is to be considered a true clinically significant difference in compositionality. For instance, it may be that a select few pathogens have a large influence in some individuals, but not in others. This may be undetected when examining differences on group levels.

An intriguing finding in our study was that *Moraxella*, a pathogen known to be involved in COPD exacerbations, was present in high abundance in stable-state COPD in five participants. Four of these participants were in the exacerbation group. This was also reflected by differences in β diversity (weighted UniFrac distance) separating these samples from the remaining samples. The participant presenting with *Moraxella* without exacerbation in the follow-up was not part of the diversity analyses due to low sampling depth (below 1000). Although the presentation of *Moraxella* is not useful for predicting exacerbations in the group as a whole, we might speculate that this alteration in the microbiota can have a clinical impact on these individuals.

Overall, our results challenge previous findings of alterations in the stable microbiota in patients with different exacerbation frequencies. However, results are difficult to compare across studies due to variations in sampling technique (induced or spontaneous sputum or bronchoscopically sampled BAL or brushes), DNA extraction, choice of primers and hypervariable region, number of PCR cycles, management of negative controls, bioinformatic processing and statistical analysis. Each step of the microbiome analysis workflow can introduce bias, for instance by over-identifying certain bacterial taxa. This limits comparison of results from different protocols, and even misrepresents the true distribution within a study [34].

There are some methodological limitations in our study as well: 1) We have relied on self-reported information regarding exacerbations and medication use. Even though participants were called four times throughout the 1-year follow-up period, this can be prone to recall bias. 2) We do not present quantitative results. Quantitative PCR was performed on a subset of samples in MicroCOPD as part of a method paper on laboratory contamination [35], but the extent of this analysis was not sufficient to evaluate whether bacterial load was different between groups in this analysis. Pragman *et al.* [14] found no difference in bacterial load in groups of different exacerbation frequencies. However, it is biologically plausible that differences in bacterial load by itself impacts the immune response, and our study cannot assess this. 3) Our analyses are based on one sampling of the microbiota, at one time point for each participant. Each sample's representativeness for an individual's microbiota is uncertain, as we do not know how stable the microbiota is. Mayhew *et al.* [8] reported that stability of sputum microbiota over time was more likely to be decreased in individuals with higher exacerbation frequencies. Due to the cross-sectional sampling, this was not possible to assess in the current study. 4) The MicroCOPD study included many samples from a large number of participants. Although handled by only three well-trained laboratory technicians at our centre, samples had to be run in different batches. We looked for batch effects by examining principal-component analysis plots of different β diversity metrics, which showed no clustering based on run (see supplementary material). Samples from participants with exacerbations *versus* without were not unevenly distributed across runs.

The mentioned shortcomings are widely shared with published studies in the field. However, we have also applied some methods that strengthen the integrity of our results. First, MicroCOPD is the largest bronchoscopy study on the COPD microbiota. Second, we performed prospective follow-up of exacerbations. Third, we argue that protected sampling by bronchoscopy is superior to sputum collection [36]. Sputum is likely contaminated as it passes through the mouth. In addition, we present OW samples in this analysis. Fourth, we sequenced negative control samples from every examination and then used the validated Decontam method [24] to identify contaminants. A paper describing the use and implications of Decontam in the MicroCOPD study is recently published [35]. We consider this statistical approach an improvement compared to other strategies in this field such as removal of all ASVs found (consistently) in negative controls [37], removal based on lists of "known" contaminants [10, 38, 39], figure presentation of negative control taxa [33], or ruling out contamination if negative samples yield no band on gel electrophoresis [40]. Other publications do not address contamination [11, 12].

Thus, in the largest study to date of the potential predictive value of the lower airway microbiota, a significant prognostic factor for later exacerbations was not found. This study highlights the difficulties in examining microbial dysbiosis of the low biomass airway microbiota in participants where the between-individual variation is potentially much larger than the between-clinical group variation. New statistical methods, or much larger sample sizes, are likely needed to overcome this problem.

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39 Wang Z, Maschera B, Lea S, *et al.* Airway host-microbiome interactions in chronic obstructive pulmonary disease. *Respir Res* 2019; 20: 113.
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# Supplementary material

## The airway microbiota and exacerbations of COPD

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## Methodological considerations

*Collection of the microbial samples.*

In the current study, we explore the lower airways microbiome by two different sampling methods, sterile brushes and protected broncheoalveolar lavage (BAL). Both methods have advantages and weaknesses, and therefore may enhance each other when both are employed.

When a bronchoscope is inserted through the upper airways, invariably the tip of the bronchoscope will come into contact with the upper airways' microbiome. We employed no suction prior to placement with the bronchoscope below the vocal cords, yet even though there is no 100% avoidance of contaminating the working channel from the microbiome that may have attached itself on the tip of the bronchoscope during the passage through the upper airways.

However, the protected specimen brushes will be truly sterile if handled correctly, since they have a wax sealed tip which can by released once the brush is visually in safe distance from the bronchoscope tip, and then be applied by visual inspection in the bronchi. The brush shall then be retracted to safe distance within their plastic sheet covering by visual guidance before pulling out, and the brush tip must be cut by sterile scissors into a sterile tube, as was done in the current study, and rarely reported upon in detail by others. The main problem with the brushes is that the area sampled is distinct but small, and the biomass per one brush tends to be very small. To mitigate this, we used three brushes in each patient at each sampling site, and each brush was brushed ten times back and forth without excessive force, at each sampling site.

Sampling BAL offers the advantage of obtaining microbial biomass from a much larger geographical area of a bronchial tree, and also, unrelated to the current analyses, the potential for fluid for biomarker analyses and differential cell counts.

However, BAL have contamination issues, especially if one merely installs the fluid through the working channel, likely contaminated by previous suctioning, and not usually fully sterile by regular scope washing procedures. We used a sterile (Combicath) inner catheter with a sealed tip to install the fluid. Even though, our BAL procedure can still not be 100% sterile by design, since when the fluid is instilled, some minute amounts of fluid will touch the tip of the bronchoscope, and thus possibly be contaminated. Another potential issue with BAL is yield. Although a controlled similar amount of fluid may be installed, yield will vary between patients. Especially in patients with a large degree of emphysema, much more fluid is "lost" to the periphery, and not returned. How to correct for this is uncertain, as the effects on the concentration of microbial mass is uncertain. Like other researchers before us, we choose not to attempt any adjustment for yield. This potential problem is presumably absent with the use of brushes, however there is invariably variation of force and length of each brushing, for each patient and each bronchoscopist, illustrating that no sampling method can be 100% standardized.

*Control samples*

Each bronchoscopy day, we opened a new, sealed, sterile 500mL Phosphate Buffered Saline (PBS) bottle. From this bottle, PBS was drawn with sterile syringes, used for BAL sampling, the fluid used for Oral wash sampling, and also the fluid in which the sterile brushes were immersed in sterile tubes. Also, we collected small amounts of this fluid in sterile tubes and froze them. These latter samples were our negative control samples. Thus, the negative

controls are PBS fluid - which all other samples will have been in contact with - but clean from the sterile bottle - the very same bottle all sample fluids were drawn from. For every single procedure in each examined participant, we thus have a negative control sample available for microbial sequencing. Sequencing the negative controls enabled us to assess sequences which must be considered contaminants, since they either must have been acquired from the PBS bottle, or from the laboratory handling. These negative control samples were extremely useful for the Decontam algorithm, allowing the use of the prevalence-based method. We have previously examined in detail the possible contamination in the MicroCOPD study, showing the usefulness of these negative control samples [1].

*Further details on data curation*

All samples in our prediction of exacerbation analysis belong to the larger MicroCOPD study, collected with an identical protocol, and thus likely to see the same sampling and laboratory related contaminants, in addition to other study-wide phenomena such as index bleeding. The samples were analysed on one MiSeq instrument, however over different 30 runs due to the large number of samples (>2500). Since the quality scores will differ for the different runs, and the Divisive Amplicon Denoising Algorithm version 2 (DADA2) algorithm uses the quality scores for the denoising, DADA2 requires that each run is imported and denoised separately before merging. In addition to specifying the minimum phred score per base, the DADA2 relies on visual inspection of the quality plot of the forward and reverse reads, and then deciding where to cut the forward and reverse reads per run. Invariably, this is subjective to the eye of the beholder, and somewhat arbitrary over 30 runs.

Thus, we chose to pre-trim the sequences by the software tool Trimmomatic (v0.39) [2], using the following parameters:

*-phred33, and algorithm: HEADCROP:17:300:21:300 LEADING:20 TRAILING:25 SLIDINGWINDOW:4:20 MINLEN:220.*

The HEADCROP:17:300:21:300 ensures the primers which were 17 bp forward and 21 bp reverse were removed. Since quality was regularly lower for the very first as well as the tail end of the reads, LEADING and TRAILING were set to 20 and 25 respectively. SLIDING WINDOW represents the possibility not present in DADA2, effectively picking out low quality reads within the middle range of the reads. However, the main advantage to using Trimmomatic in our study was to ensure all runs were treated exactly the same regarding quality requirements.

After this trimming, each of the 30 runs were imported into QIIME 2 (v2018.8) and denoised with DADA2, using the command:

*qiime dada2 denoise-paired --i-demultiplexed-seqs*
*/Volumes/.../RUNX_workingfiles/RUNX_importdemux/RUNXimport-trimmed-demux.qza*
*--output-dir /Volumes/LaCie/RUN6_workingfiles/RUNX_denoisedpaired*
*--p-trunc-len-f 0 --p-trunc-len-r 0 --p-trim-left-f 0 --p-trim-left-r 0*
[already trimmed by trimmomatic]
*--p-chimera-method consensus*

And after that the files from the 30 runss were merged, before another round of chimera removal using VSEARCH.

*Handling of contamination using information from the control samples*

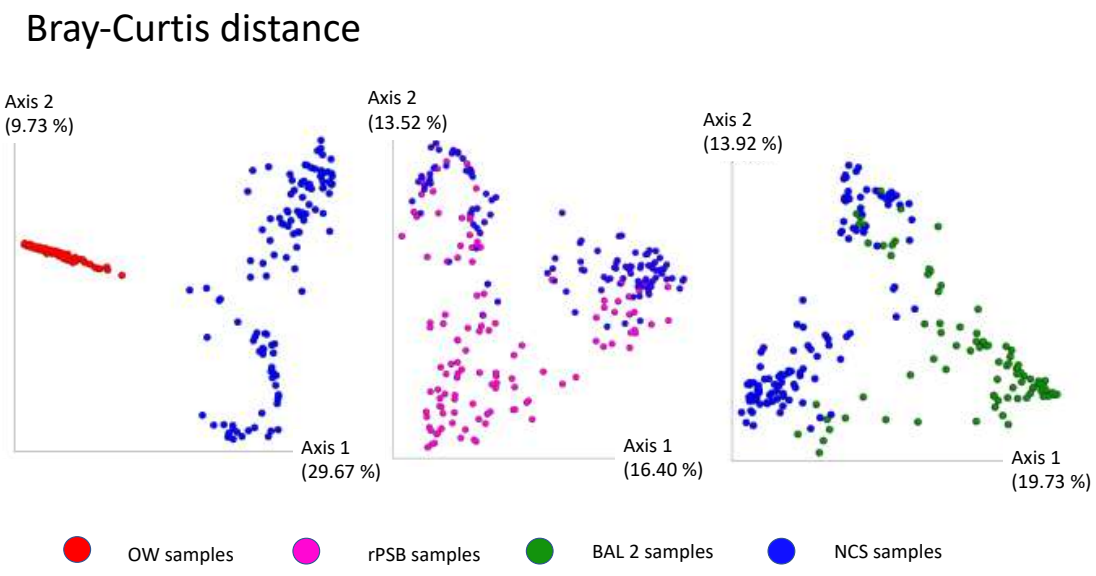
In order to do effective contamination removal, taking into account all samples in the relevant runs, we kept all samples as long as possible in the up-stream processing. After the

steps described above, the ASV table was exported for use in R (v3.4.1) with the Decontam algorithm (v1.1.2) [3].

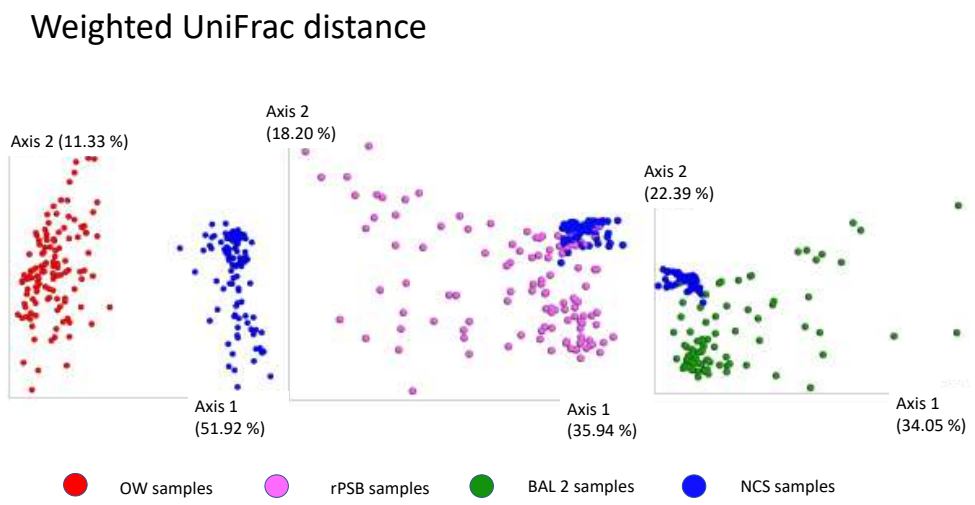
We chose the prevalence method which is adviced for low-biomass samples such as ours, and where the contaminants are identified by increased prevalence in negative controls relative to the true biological samples. A key parameter is the threshold parameter, which is the probability threshold below which a contaminant should be rejected in favor of a non-contaminant. After running the algorithm, frequency distribution plots can be run to assess how well the contaminants and non-contaminants are distinguished between each other. We ran Decontam with thresholds 0.3, 0.4 and 0.5, and found that a threshold of 0.3 struck the best balance between distinguishing likely contaminants, and retaining likeli non-contaminant reads. With threshold = 0.3, we identified 280 ASVs deemed likely contaminants.

To see how the negative controls differed from the biological samples, we provide principal coordinate analysis (PCoA) plots with Bray-Curtis, weighted and unweighted UniFrac distances, showing the clustering of samples before the Decontam step (Supplemetary figures 1-3).

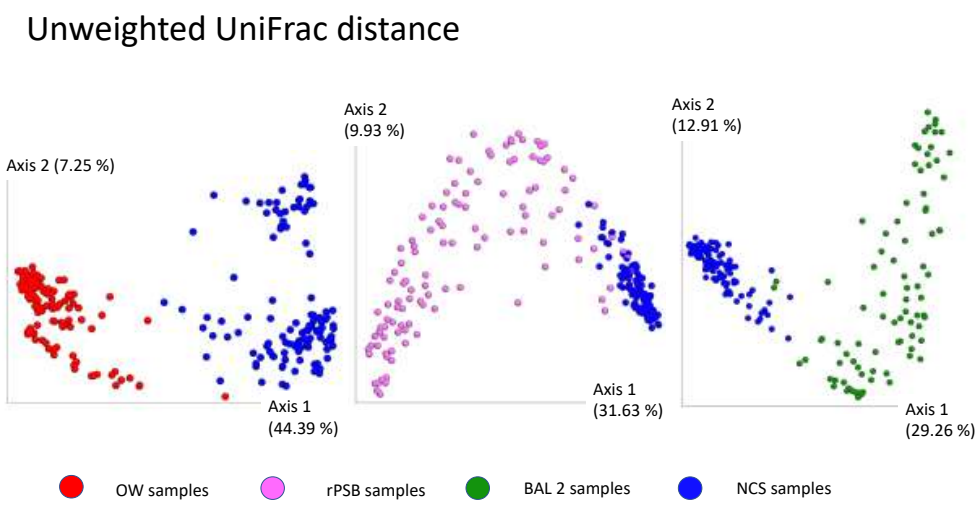
**Supplementary figure 1:** Bray-Curtis distances between airway samples and negative control samples. Samples in this figure are from the participants in this study, before the Decontam step and rarefied to a sampling depth of 1000 before diversity analyses. OW: Oral wash, rPSB: Right protected specimen brushes, BAL 2: Second fraction of bronchoalveolar lavage, NCS: Negative control samples.



**Supplementary figure 2:** Weighted UniFrac distances between airway samples and negative control samples. Samples in this figure are from the participants in this study, before the Decontam step and rarefied to a sampling depth of 1000 before diversity analyses. OW: Oral wash, rPSB: Right protected specimen brushes, BAL 2: Second fraction of bronchoalveolar lavage, NCS: Negative control samples.



**Supplementary figure 3:** Unweighted UniFrac distances between airway samples and negative control samples. Samples in this figure are from the participants in this study, before the Decontam step and rarefied to a sampling depth of 1000 before diversity analyses. OW: Oral wash, rPSB: Right protected specimen brushes, BAL 2: Second fraction of bronchoalveolar lavage, NCS: Negative control samples.




When collapsed to the genus level (not all could be assigned taxonomy at the genus level), the number of separate taxa was 82. These 82 taxa are listed below. Taxa are ordered according to their relative abundance among identified contaminants across all samples, in increasing order:

g__Neisseriaceae_[G-1]
g__Veillonellaceae_[G-1]
p__Bacteroidetes;__;__;__;__
g__Clostridiales_[F-3][G-1]
g__Lactococcus
g__Caulobacter
g__Bdellovibrio
g__Mitsuokella
f__Comamonadaceae;__
p__Firmicutes;__;__;__;__
g__Defluvibacter
g__Enhydrobacter
g__Comamonas
g__Dialister
c__Alphaproteobacteria;__;__;__
g__Bosea
g__Lactobacillus

g__Alloprevotella
p__Proteobacteria;__;__;__;__
f__Rhizobiaceae;__
o__Burkholderiales;__;__
g__Campylobacter
g__Achromobacter
g__Ochrobactrum
g__Paracoccus
g__Corynebacterium
g__Bifidobacterium
g__Moraxella
g__Microbacterium
g__Bacillus
f__Neisseriaceae;__
g__Saccharibacteria_(TM7)_[G-6]
g__Bacteroidales_[G-2]
g__Butyrivibrio
g__Arthrospira
g__Lachnospiraceae_[G-2]
g__Acinetobacter
g__Megasphaera
g__Cupriavidus
g__Micrococcus
g__Leptothrix
g__Peptostreptococcaceae_[XI][G-9]
g__Lysinibacillus
g__Cutibacterium
g__Gemella
g__Leptotrichia
c__Betaproteobacteria;__;__;__
g__Kingella
g__Roseomonas
g__Pedobacter
c__Gammaproteobacteria;__;__;__
g__Sphingomonas
g__Actinomyces
g__Stenotrophomonas
g__Mesorhizobium
g__Capnocytophaga
g__Selenomonas
o__Rhizobiales;__;__
f__Bradyrhizobiaceae;__
f__Enterobacteriaceae;__
g__Fusobacterium
k__Bacteria;__;__;__;__;__
g__Brevundimonas
g__Staphylococcus
g__Porphyromonas
c__Actinobacteria;__;__;__
g__Burkholderia
g__Agrobacterium
g__Haemophilus
f__Burkholderiaceae;__
g__Bergeyella
g__Segetibacter
g__Prevotella
g__Veillonella

g__Streptococcus
g__Rothia
g__Pseudomonas
g__Neisseria
g__Delftia
c__Negativicutes;__;__;__
g__Klebsiella
g__Ralstonia

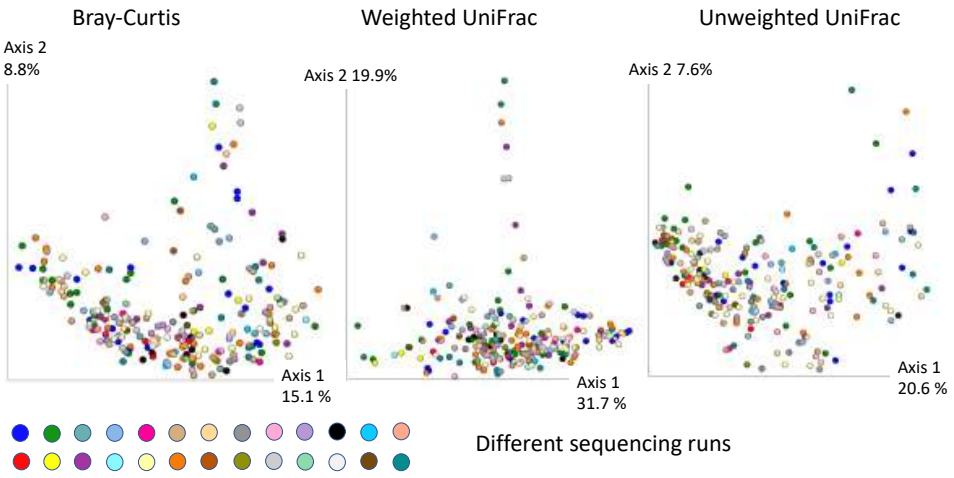
g = genus, c = class, f= family, o = order, p = phylum, k = kingdom

*Examining potential batch effects*

As mentioned above, data curation and quality criteria were similar for all 30 RUNs.

After merging and curation was finalized, we created a PCoA plot of all samples per patient to see if some samples clustered by sequencing RUN. We were unable to see clustering according to sequencing RUN (Supplementary figure 4).

**Supplementary figure 4:** PCoA plots of three different beta diversity metrices (Bray-Curtis, weighted and unweighted UniFrac distances) for all samples from the 105 participants with exacerbation follow-up after rarefaction to a sampling depth of 1000, coloured according to sequencing run.



## Additional results

*Differential abundance testing*

**Supplementary table 1.** Results from 4 different tests (ANCOM [4], gneiss [5], ALDEx2 [6], MicrobiomeDDA [7]) of differences in abundance of taxa, either at the ASV or genus level, between patients who did and did not experience one or more later COPD exacerbations.

| **Test** | **Sample type** | **Level** | **Taxonomic annotation of differentially expressed features:** | **Details** |
|---|---|---|---|---|
| ANCOM | BAL | ASV | No differentially abundant features | |
| | | Genus | No differentially abundant genera | |
| | rPSB | ASV | No differentially abundant features | |
| | | Genus | No differentially abundant genera | |
| gneiss | BAL | ASV | No significant balances | |
| | rPSB | ASV | No significant balances | |
| ALDEx2 | BAL | ASV | No differentially abundant features | |
| | | Genus | No differentially abundant genera | |
| | rPSB | ASV | No differentially abundant features | |
| | | Genus | No differentially abundant genera | |
| MicrobiomeDDA | BAL | ASV | No differentially expressed features | |
| | | Genus | No differentially expressed genera | |
| | rPSB | ASV | *Capnocytophaga gingivitis*<br>(more abundant, less prevalent and less dispersed in group with exacerbations)<br><br>*Prevotella pallens*<br>(less abundant, more prevalent and more dispersed in group with exacerbations) | abund.LFC: 1.693,<br>prev.change: -0.054<br>disp.LFC: -3.301<br>statistic: 20.035<br>P.adj: 0.011<br><br>abund.LFC: -0.601<br>prev.change: 0.060<br>disp.LFC: 28.006<br>statistic: 15.627<br>P.adj: 0.041 |
| | | Genus | No differentially abundant genera | |

Abund.LFC: log2-fold change in fitted mean abundance parameter between exacerbation and no-exacerbation group. Prev.change: linear difference in prevalence between exacerbation and no-exacerbation group. Disp.LFC: log2-fold change in fitted dispersion parameter between exacerbation and no-exacerbation group. Statistic: Value of likelihood ratio test statistic. Padj: p-value adjusted for multiple comparisons according to the FDR/BH method.

**Supplementary table 2.** Results from 4 different tests (ANCOM [4], gneiss [5], ALDEx2 [6], MicrobiomeDDA [7]) of differences in abundance of taxa, either at the ASV or genus level, between patients who had none or one and patients who experienced two or more later COPD exacerbations.

| **Test** | **Sample type** | **Level** | **Taxonomic annotation of differentially expressed features:** | **Details** |
|---|---|---|---|---|
| ANCOM | BAL | ASV | No differentially abundant features | |
| | | Genus | No differentially abundant genera | |
| | rPSB | ASV | No differentially abundant features | |
| | | Genus | No differentially abundant genera | |
| gneiss | BAL | ASV | No significant balances | |
| | rPSB | ASV | No significant balances | |
| ALDEx2 | BAL | ASV | No differentially abundant features | |
| | | Genus | No differentially abundant genera | |
| | rPSB | ASV | No differentially abundant features | |
| | | Genus | No differentially abundant genera | |
| MicrobiomeDDA | BAL | ASV | *Capnocytophaga leadbetteri*<br>(less abundant, more prevalent and less dispersed in group with two or more exacerbations) | abund.LFC: -2.462<br>prev.change: 0.052<br>disp.LFC: -2.155<br>statistic: 24.360<br>P.adj: 0.008 |
| | | | *Prevotella oris*<br>(less abundant, more prevalent and more dispersed in group with two or more exacerbations) | abund.LFC: -3.424<br>prev.change: 0.0004<br>disp.LFC: 1.826<br>statistic: 19.169<br>P.adj: 0.045 |
| | | Genus | *Moraxella*<br>(less abundant, more prevalent and less dispersed in group with two or more exacerbations) | abund.LFC: 4.076<br>prev.change: 0.175<br>disp.LFC: 27.53<br>statistic: 18.215<br>P.adj: 0.026 |
| | rPSB | ASV | No differentially expressed features | |
| | | Genus | No differentially expressed genera | |

Abund.LFC: log2-fold change in fitted mean abundance parameter between exacerbation and no-exacerbation group. Prev.change: linear difference in prevalence between exacerbation and no-exacerbation group. Disp.LFC: log2-fold change in fitted dispersion parameter between exacerbation and no-exacerbation group. Statistic: Value of likelihood ratio test statistic. Padj: p-value adjusted for multiple comparisons according to the FDR/BH method.

**Supplementary table 3.** Results from 4 different tests (ANCOM, gneiss, ALDEx2, MicrobiomeDDA) of differences in abundance of taxa, either at the ASV or genus level, between COPD patients who used and did not use inhaled corticosteroids (ICS) at baseline.

| **Test** | **Sample type** | **Level** | **Taxonomic annotation of differentially expressed features:** | **Details** |
|---|---|---|---|---|
| ANCOM | BAL | ASV | No differentially abundant features | |
| | | Genus | No differentially abundant genera | |
| | rPSB | ASV | No differentially abundant features | |
| | | Genus | No differentially abundant genera | |
| gneiss | BAL | ASV | No significant balances | |
| | rPSB | ASV | No significant balances | |
| ALDEx2 | BAL | ASV | No differentially abundant features | |
| | | Genus | No differentially abundant genera | |
| | rPSB | ASV | No differentially abundant features | |
| | | Genus | No differentially abundant genera | |
| MicrobiomeDDA | BAL | ASV | No differentially expressed features | |
| | | Genus | No differentially expressed genera | |
| | rPSB | ASV | No differentially expressed features | |
| | | Genus | No differentially expressed genera | |
| Abund.LFC: log2-fold change in fitted mean abundance parameter between ICS usage and no ICS usage group. Prev.change: linear difference in prevalence between ICS usage and no ICS usage group. Disp.LFC: log2-fold change in fitted dispersion parameter between ICS usage and no ICS usage group.<br>Statistic: Value of likelihood ratio test statistic. Padj: p-value adjusted for multiple comparisons according to the FDR/BH method. | | | | |

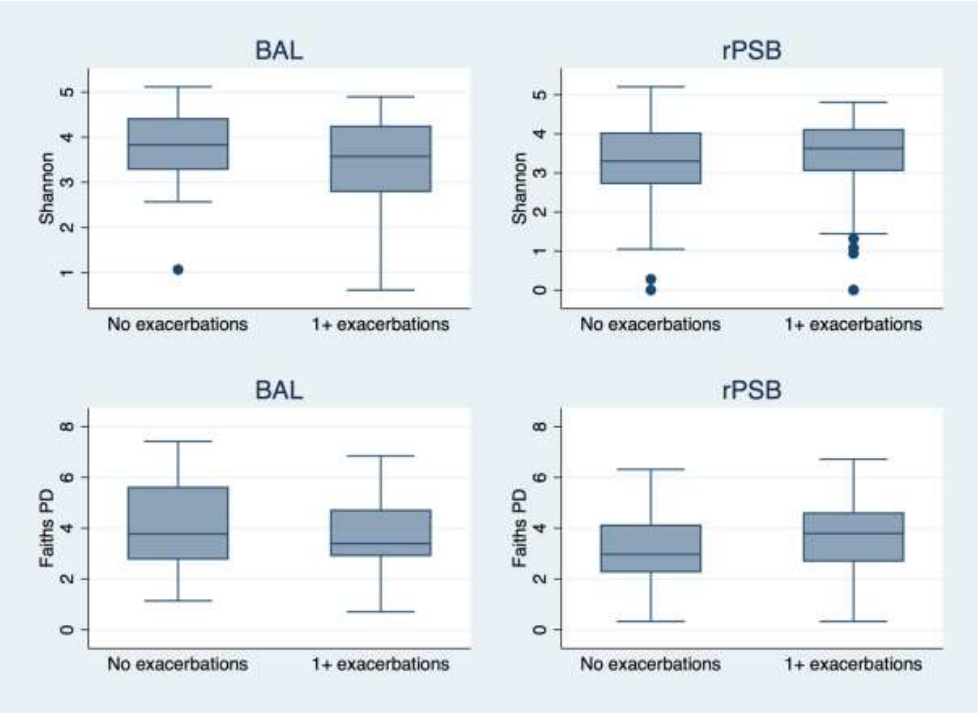
**Supplementary table 4.** Results from 4 different tests (ANCOM [4], gneiss [5], ALDEx2 [6], MicrobiomeDDA [7]) of differences in abundance of taxa, either at the ASV or genus level, between patients who did and did not experience one or more COPD exacerbations in the 12 months prior to the bronchoscopy.

| **Test** | **Sample type** | **Level** | **Taxonomic annotation of differentially expressed features:** | **Details** |
|---|---|---|---|---|
| ANCOM | BAL | ASV | No differentially abundant features | |
| | | Genus | No differentially abundant genera | |
| | rPSB | ASV | No differentially abundant features | |
| | | Genus | No differentially abundant genera | |
| gneiss | BAL | ASV | No significant balances | |
| | rPSB | ASV | No significant balances | |
| ALDEx2 | BAL | ASV | No differentially abundant features | |
| | | Genus | No differentially abundant genera | |
| | rPSB | ASV | No differentially abundant features | |
| | | Genus | No differentially abundant genera | |
| MicrobiomeDDA | BAL | ASV | *Actinomyces graevenitzii* (less abundant, more prevalent and less dispersed in patients having experienced exacerbations) | abund.LFC: -1.513<br>prev.change: 0.028<br>disp.LFC: -0.618<br>statistic: 23.660<br>P.adj: 0.011 |
| | | Genus | No differentially expressed genera | |
| | rPSB | ASV | No differentially expressed features | |
| | | Genus | No differentially expressed genera | |

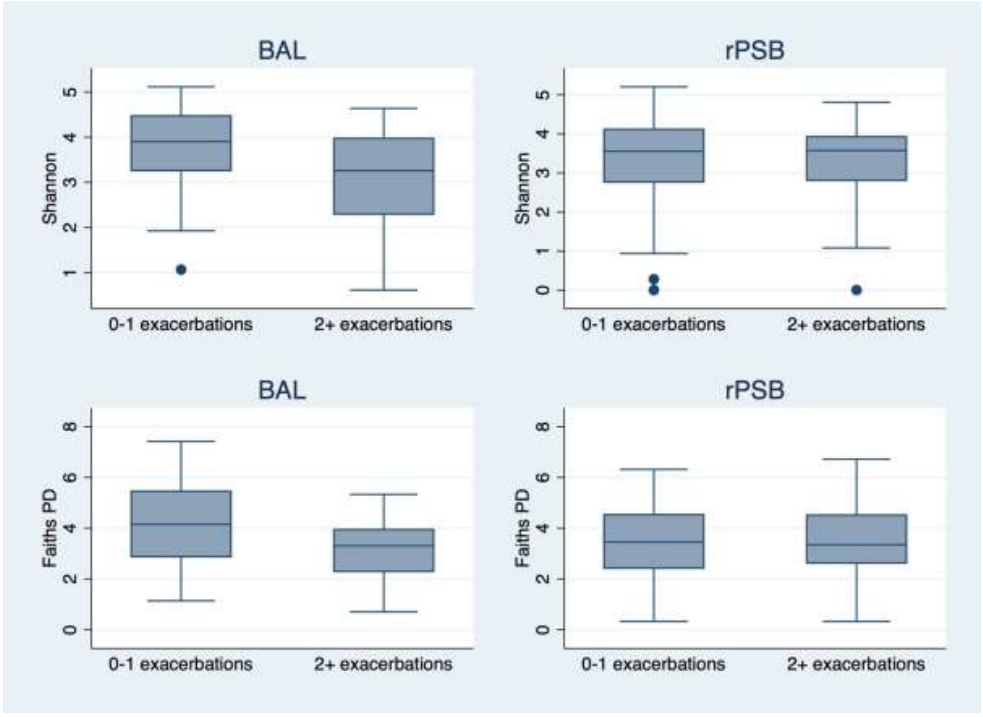
Abund.LFC: log2-fold change in fitted mean abundance parameter between exacerbation and no-exacerbation group. Prev.change: linear difference in prevalence between exacerbation and no-exacerbation group. Disp.LFC: log2-fold change in fitted dispersion parameter between exacerbation and no-exacerbation group. Statistic: Value of likelihood ratio test statistic. Padj: p-value adjusted for multiple comparisons according to the FDR/BH method.

*Diversity analyses*

**Supplementary figure 5:** Box plots of Faith phylogenetic alpha diversity (Faith PD) and Shannon Index alpha diversity in patients without compared with one or more later exacerbations in 60 bronchoalveolar lavage (BAL2) and 73 protected specimen brush samples (rPSB). Differences in diversity were tested with Kruskal-Wallis. There were no statistically significant associations.



**Supplementary figure 6:** Box plots of Faith phylogenetic alpha diversity (Faith PD) and Shannon Index alpha diversity in patients zero or one compared with two or more later exacerbations in 60 bronchoalveolar lavage (BAL2) and 73 protected specimen brush samples (rPSB). Differences in diversity were tested with Kruskal-Wallis. There were no statistically significant associations.



**Supplementary table 5.** Permutational multivariate analysis of variance (PERMANOVA [8]) of the beta-diversity (weighted UniFrac) by exacerbation category (zero versus one or more) without and with adjustment for age, sex, $FEV_1$ and use of inhaled steroids, in bronchoalveolar lavage samples and right protected specimen brush samples. Analysed with the vegan package in R.

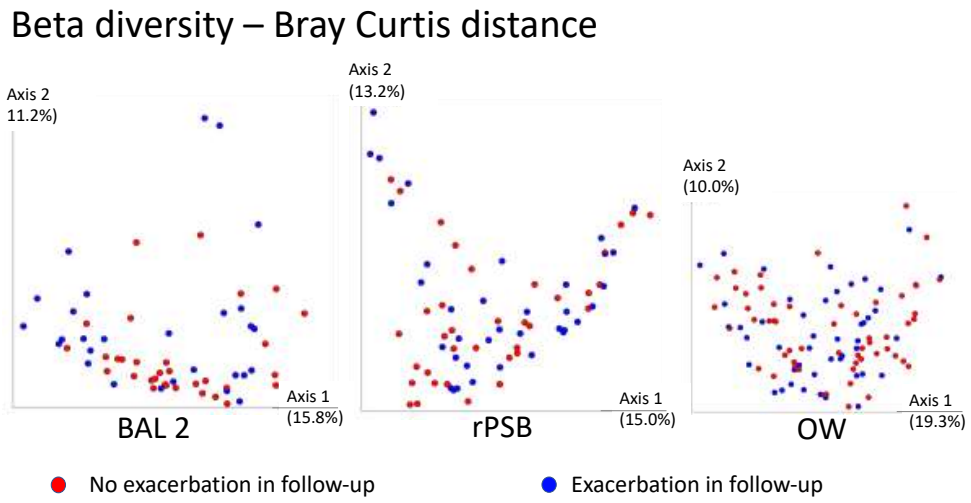
| *Bronchoalveolar Lavage* | | | | | | |
|---|---|---|---|---|---|---|
| | Df | SumsOfSqs | MeanSqs | F.Model | R2 | Pr(>F) |
| Exacerbation category | 1 | 0.04811 | 0.048107 | 1.173 | 0.01982 | 0.287 |
| Residuals | 58 | 2.37876 | 0.041013 | | 0.98018 | |
| | | | | | | |
| | Df | SumsOfSqs | MeanSqs | F.Model | R2 | Pr(>F) |
| Exacerbation category | 1 | 0.04811 | 0.048107 | 1.15092 | 0.01982 | 0.324 |
| Age | 1 | 0.01602 | 0.016016 | 0.38316 | 0.00660 | 0.898 |
| Sex | 1 | 0.04037 | 0.040369 | 0.96578 | 0.01663 | 0.416 |
| FEV1 in percent predicted | 1 | 0.02836 | 0.028357 | 0.67840 | 0.01168 | 0.618 |
| Inhaled steroid use | 1 | 0.03686 | 0.036863 | 0.88190 | 0.01519 | 0.507 |
| Residuals | 54 | 2.25715 | 0.041799 | | 0.93007 | |
| | | | | | | |
| *right Protected Specimen Brush* | | | | | | |
| | Df | SumsOfSqs | MeanSqs | F.Model | R2 | Pr(>F) |
| Exacerbation category | 1 | 0.0518 | 0.051754 | 0.88513 | 0.01231 | 0.462 |
| Residuals | 71 | 4.1514 | 0.058470 | | 0.98769 | |
| | | | | | | |
| | Df | SumsOfSqs | MeanSqs | F.Model | R2 | Pr(>F) |
| Exacerbation category | 1 | 0.0518 0.051754 | 0.9192 | 0.01231 | 0.414 | |
| Age | 1 | 0.1884 | 0.188388 | 3.3458 | 0.04482 | |
| Sex | 1 | 0.0395 | 0.039534 | 0.7021 | 0.00941 | 0.589 |
| FEV1 in percent predicted | 1 | 0.0834 0.083391 | 1.4810 | 0.01984 | 0.188 | |
| Inhaled steroid use | 1 | 0.0676 | 0.067642 | 1.2014 | 0.01609 | 0.294 |
| Residuals | 67 | 3.7724 | 0.056305 | | 0.89753 | |

**Supplementary table 6.** Permutational multivariate analysis of variance (PERMANOVA [8]) of the beta-diversity (weighted UniFrac) by exacerbation category (zero or one versus two or more) without and with adjustment for age, sex, $FEV_1$ and use of inhaled steroids, in bronchoalveolar lavage samples and right protected specimen brush samples. Analysed with the vegan package in R.

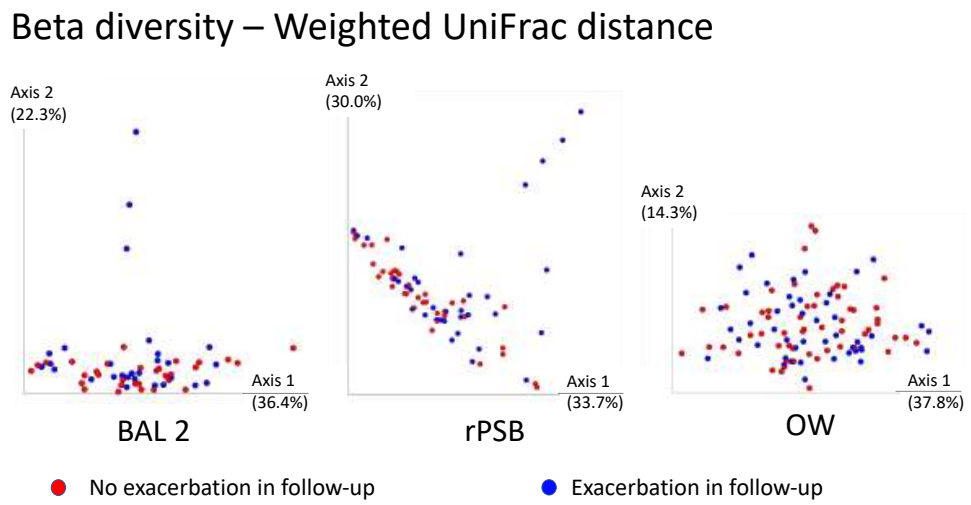
| *Bronchoalveolar Lavage** | | | | | | |
|---|---|---|---|---|---|---|
| | Df | SumsOfSqs | MeanSqs | F.Model | R2 | Pr(>F) |
| Exacerbation category | 1 | 0.1078 | 0.107837 | 2.64261 | 0.04358 | 0.031 |
| Residuals | 58 | 2.3668 | 0.040807 | | 0.95642 | |
| | | | | | | |
| | Df | SumsOfSqs | MeanSqs | F.Model | R2 | Pr(>F) |
| Exacerbation category | 1 | 0.1078 | 0.107837 | 2.59727 | 0.04358 | 0.031 |
| Age | 1 | 0.0622 | 0.062161 | 1.49716 | 0.02512 | 0.172 |
| Sex | 1 | 0.0199 | 0.019898 | 0.47925 | 0.00804 | 0.822 |
| FEV1 in percent predicted | 1 | 0.0223 | 0.022272 | 0.53643 | 0.00900 | 0.763 |
| Inhaled steroid use | 1 | 0.0204 | 0.020431 | 0.49208 | 0.00826 | 0.800 |
| Residuals | 54 | 2.2420 | 0.041519 | | 0.90601 | |
| | | | | | | |
| *right Protected Specimen Brush* | | | | | | |
| | Df | SumsOfSqs | MeanSqs | F.Model | R2 | Pr(>F) |
| Exacerbation category | 1 | 0.2154 | 0.215415 | 3.83368 | 0.05123 | 0.006 |
| Residuals | 71 | 3.9895 | 0.056190 | | 0.94877 | |
| | | | | | | |
| | Df | SumsOfSqs | MeanSqs | F.Model | R2 | Pr(>F) |
| Exacerbation category | 1 | 0.2154 | 0.215415 | 3.85941 | 0.05123 | 0.007 |
| Age | 1 | 0.0886 | 0.088620 | 1.58772 | 0.02108 | 0.169 |
| Sex | 1 | 0.0263 | 0.026253 | 0.47036 | 0.00624 | 0.788 |
| FEV1 in percent predicted | 1 | 0.0733 | 0.073289 | 1.31306 | 0.01743 | 0.248 |
| Inhaled steroid use | 1 | 0.0617 | 0.061702 | 1.10546 | 0.01467 | 0.329 |
| Residuals | 67 | 3.7396 | 0.055815 | | 0.88935 | |

* For Broncheoalveolar lavage the dispersion was significant, and the results cannot be trusted, the results are shown only for consistency with supplementary table 5.

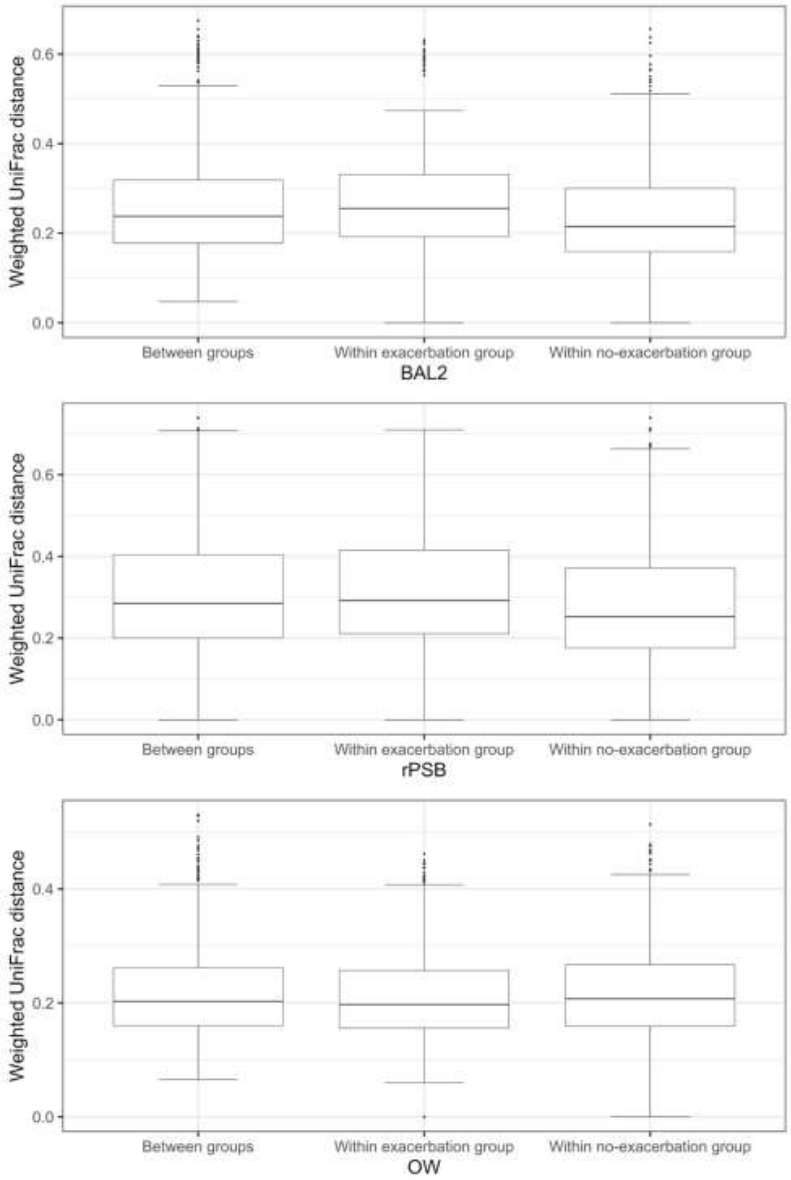
**Supplementary figure 7:** Principle Coordinate plots of beta diversity, measured by Bray Curtis distance in second fraction of bronchoalveolar lavage (BAL2), protected specimen brush (rPSB) and oral wash (OW) samples. Each sample is coloured according to exacerbation status (zero versus one or more) during follow-up.




**Supplementary figure 8:** Principle Coordinate plots of beta diversity, measured by weighted UniFrac distance in second fraction of bronchoalveolar lavage (BAL2), protected specimen brush (rPSB) and oral wash (OW) samples. Each sample is coloured according to exacerbation status (zero versus one or more) during follow-up.



**Supplementary figure 9:** Beta diversity, measured by weighted UniFrac distance. Box plots of distances between groups with and without later exacerbations for each sample type; oral wash (OW), right protected specimen brushes (rPSB) and second fraction of bronchoalveolar lavage (BAL2). Differences between all groups were tested with the ADONIS permutation-based test. There were no statistically significant differences.



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