“We live on an island surrounded by a sea of ignorance. As our island of knowledge grows, so does the shore of our ignorance.”

John Archibald Wheeler

DANKWOORD

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LIST OF ABBREVIATIONS

% pred	Percentage predicted
ABPA	Allergic bronchopulmonary aspergillosis
ACO	Asthma-COPD overlap
ACT	Asthma control test
A. fumigatus	*Aspergillus fumigatus*
BD	Bronchodilator
BMI	Body mass index
CAT	COPD assessment test
CF	Cystic fibrosis
CI	Confidence interval
COPD	Chronic Obstructive Pulmonary Disease
CS	Corticosteroids
CT	Computed tomography
DLCO	Diffusion capacity of the lung for carbon monoxide
F	Female
FEF25-75%	Forced expiratory flow between 25-75%
FeNO	Fractional exhaled nitric oxide
FEV1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GOLD	Global Initiative for Chronic Obstructive Lung Disease
HRCT	High resolution computed tomography
ICS	Inhaled corticosteroids
IQR	interquartile range
IgE	Immunoglobulin E
IgG	Immunoglobulin G
KCO	Carbon monoxide transfer coefficient
LLN	Lower limit of normal
M	Male

mMRC	modified Medical Research Council breathlessness scale
NTM	Nontuberculous mycobacteria
OR	Odds ratio
PPM	Potential pathogenic micro-organisms
rAsp f	Recombinant antigen of *Aspergillus fumigatus*
RV	Residual volume
TB	Terminal bronchioles
TGV	Thoracic gas volume
Th2	T helper type 2
TLC	Total lung capacity
tSNE	T-distributed stochastic neighbor embedding
VAS	Visual analogue scale

Chapter 1

General introduction

1. Chronic Obstructive Pulmonary Disease

Chronic Obstructive Pulmonary Disease (COPD) is defined as a common, preventable and treatable disease that is characterized by persistent respiratory symptoms and airflow limitation, due to airway and/or alveolar abnormalities, which are usually caused by significant exposure to noxious particles or gases (1). Cigarette smoke is the main culprit in developed countries, whereas indoor exposure to smoke from biomass fuel is an important cause in low-income countries. Risk factors for the development of COPD include age, early life respiratory events, physical inactivity and exposure to pollutants, next to genetic and epigenetic factors (2,3). Nevertheless, many questions regarding predisposition to the disease remain unanswered.

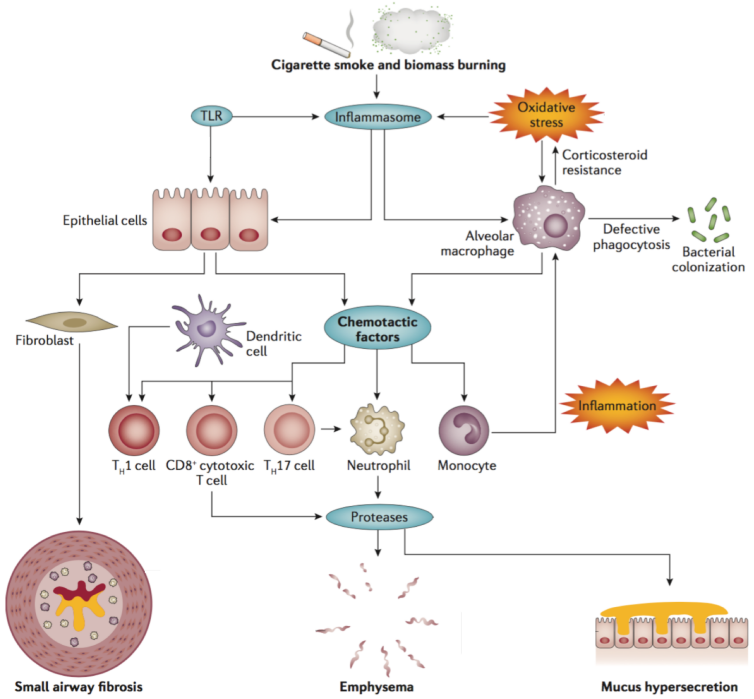
COPD is a highly prevalent disease, given a worldwide prevalence of 5 to 15%, with an estimated prevalence of 5 to 8% in Belgium. A global increase in the burden of COPD is expected, due to continued exposure to COPD risk factors and aging of the population. Next to the high prevalence, both morbidity and mortality are high. COPD kills more than 3 million people worldwide every year and is predicted by the World Health Organization to become the third leading cause of death by 2030 (1,4). Moreover, COPD was globally in the top 10 of causes for lost Disability-Adjusted Life Years in 2016 (5). Consequently, the social and economic burden of this disease is huge.

1.1 Pathogenesis and pathophysiology

An abnormal inflammatory response is the main feature in the pathogenesis of COPD. Noxious particles may injure airway epithelial cells, leading to release of molecules, which are then recognized by pattern recognition receptors. This results in the release of cytokines, which attract macrophages, neutrophils and Tc1, Th1, Th17 and ILC3 lymphocytes to the site of inflammation. Together with structural cells, these inflammatory cells release cytokines, mediators and proteases. Increased mucus secretion and mucous hyperplasia occur at the level of the epithelial cells. Oxidative stress and impaired function of alveolar macrophages contribute to inflammation and tissue damage. (Figure 1) (3,6) Formation of lymphoid aggregates surrounding small airways, has been described in progressive disease (7).

Furthermore, accelerated aging of the lung, caused by impaired immune regulation, plays also a role in pathogenesis (8).

Figure 1: Pathogenesis of COPD



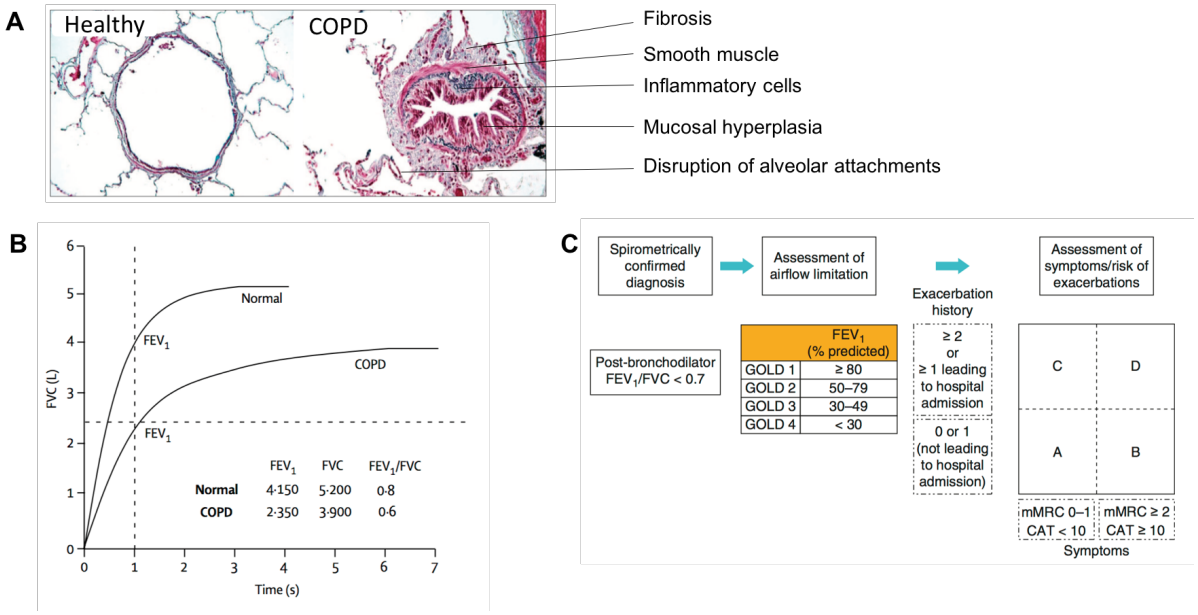
Summary of basic pathogenic mechanisms in COPD, resulting in small airway disease and emphysema. Adapted with permission (9).

Together, these inflammatory processes cause disturbed tissue repair with fibrosis, narrowing and even loss of small airways resulting in obstructive bronchiolitis (7,10). Regarding the lung parenchyma, inflammation results in damaged alveoli and loss of alveolar attachments (emphysema) which reduces elastic recoil (11)(Figure 1 and 2A). Although the relative contribution varies from person to person, both small airway and parenchymal damage cause irreversible and often progressive airflow limitation with inability of emptying the lungs on expiration. This results in static and dynamic hyperinflation of which the latter keeps the patients from physical activity (12). The mechanisms by which inflammation persists, even after discontinuation of smoking, have not yet been elucidated. Local inflammation is associated with systemic inflammation and strengthens further during exacerbations (13). Colonization with bacteria, such as *Streptococcus pneumoniae* and *Haemophilus influenzae*, is probably a consequence of reduced phagocytosis by macrophages and may drive airway and systemic inflammation (14).

1.2 Clinical aspects

Patients with COPD may suffer from dyspnea, cough, sputum production, wheezing and chest tightness. If a history of exposure to noxious stimuli is present in combination with these symptoms, COPD should be considered and confirmed by spirometry. Irreversible airflow limitation is required for the diagnosis and demonstrated by a post-bronchodilator forced expiratory volume in 1 second over forced vital capacity (FEV1/FVC) below 0.7 (Figure 2B).

Figure 2: Histopathological characteristics and diagnosis of COPD



(A) Histopathological image of a small airway from a healthy subject and from a patient with COPD, adapted with permission (15). (B) Spirometry of a subject with normal lungs and of a patient with COPD, reproduced with permission (16). (C) Proposal for assessment of stable patients with COPD from the Global initiative of Obstructive Lung Disease report, reproduced with permission (1).

The course of the disease is mostly characterized by exacerbations and comorbidities. Exacerbations are the acute worsening of respiratory symptoms, resulting in additional therapy. They may be triggered by several factors, most frequently viral respiratory tract infections. (17,18) A systematic assessment of the disease, based on degree of airflow limitation, symptoms and risk of exacerbations is proposed by the Global initiative of Obstructive Lung Disease (GOLD) to understand the individual impact of the disease and guide treatment (1) (Figure 2C). Comorbidities contribute substantially to the condition of a patient with COPD (19). Cardiovascular disorders are frequent, next to osteoporosis, anxiety and depression, lung cancer, metabolic syndrome and diabetes, gastroesophageal reflux and

obstructive sleep apnea. Prognosis of the disease is variable and influenced by multiple factors such as exacerbations, FEV1, body mass index, exercise capacity and severity of dyspnea (20).

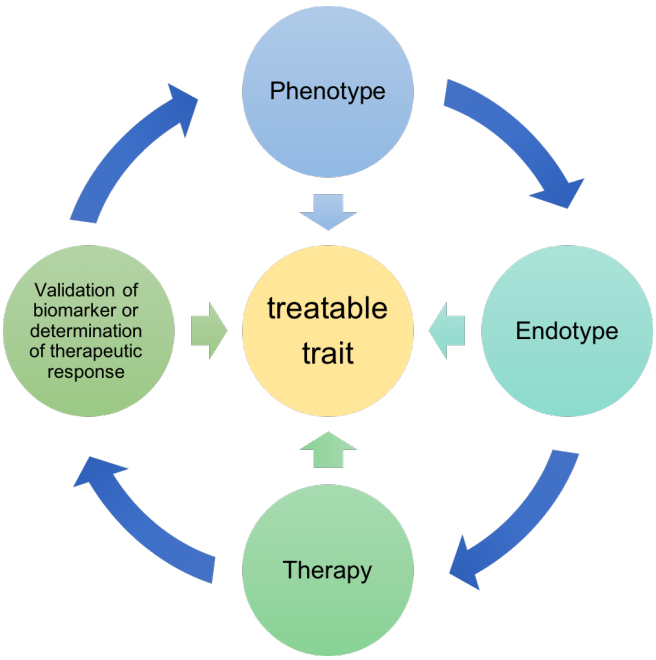
Smoking cessation is the main treatment, individualized pharmacologic therapy comes second. This includes inhalation therapy with bronchodilators (bèta-agonists and muscarinic antagonists) and corticosteroids (CS), roflumilast and macrolides. Furthermore, non-pharmacologic therapy such as vaccination, oxygen therapy, ventilatory support, pulmonary rehabilitation and surgical or bronchoscopic interventions are indicated for certain patients. Exacerbations have to be treated appropriately with intensification of inhalation therapy, systemic CS and antibiotics when indicated.

1.3 Phenotypes

COPD is a very complex and heterogeneous disorder with substantial differences in terms of clinical presentation, imaging, physiology, therapy response and disease progression. In recent years, the interest in phenotyping the disease to personalize patient management grew considerably (21). A COPD phenotype has been defined as a single or combination of disease attributes that describe differences between individuals with COPD, as they relate to clinically meaningful outcomes (symptoms, exacerbations, response to therapy, rate of disease progression or death) (22). However, phenotypes may overlap in clinical practice, further strengthening the complexity of the disease. Research into the biological mechanisms underlying clinical phenotypes, the so-called endotypes, may help in the determination of treatable traits (21,23) and development of new therapeutic strategies (Figure 3). Another element in this process is the concept of biomarkers, defined as characteristics that are objectively measured and evaluated as indicators of normal biological processes, pathogenic processes or biological responses to a therapeutic intervention (24). These are crucial in recognition of treatable traits. Despite the considerable progress made in understanding and treating COPD, current therapeutic strategies have only limited effect on the evolution of the disease. A better insight in phenotypes, endotypes and treatable traits is relevant to provide precision medicine (25). So far, only three phenotypes were validated against clinical outcome or treatment response; alpha-1 antitrypsin deficiency, a genetic condition that

predisposes to early onset of pulmonary emphysema and airway obstruction, upper-lobe predominant emphysema and frequent exacerbators (26). Nevertheless, many other clinically meaningful phenotypes have been proposed. In this thesis, two recently suggested phenotypes were investigated. Firstly, COPD-bronchiectasis overlap and particularly the role of *A. fumigatus* in this subgroup of patients. And secondly, we attempted to differentiate and characterize patients with asthma-COPD overlap as a distinct phenotype in obstructive lung disease.

Figure 3: Interplay in phenotyping process



A clinical phenotype, which is characterized by particular symptoms, physiologic or radiologic features, may result in the discovery of underlying cellular or molecular pathways or endotypes. These endotypes are targets for (development of) therapy. Presence of therapeutic response or similar outcome validates this process. All steps in this process may contribute to the recognition of treatable traits. (21–23,25)

2. Bronchiectasis

Bronchiectasis is a chronic airway disease defined by permanently dilated and chronically inflamed bronchi that repeatedly undergo infectious episodes. This translates into a clinical syndrome of cough, sputum production and recurrent respiratory infections. Bronchiectasis represents a heterogeneous group since various causes may lead to this disease, such as cystic fibrosis (CF), immune deficiencies, bronchial obstruction or respiratory infections. Although the list of underlying causes is extensive, also idiopathic bronchiectasis is common (27). The worldwide prevalence is unknown, but recent data from the United Kingdom

estimate the prevalence of non-cystic fibrosis bronchiectasis at 0,49% for men and 0,57% for women, across all age groups (28).

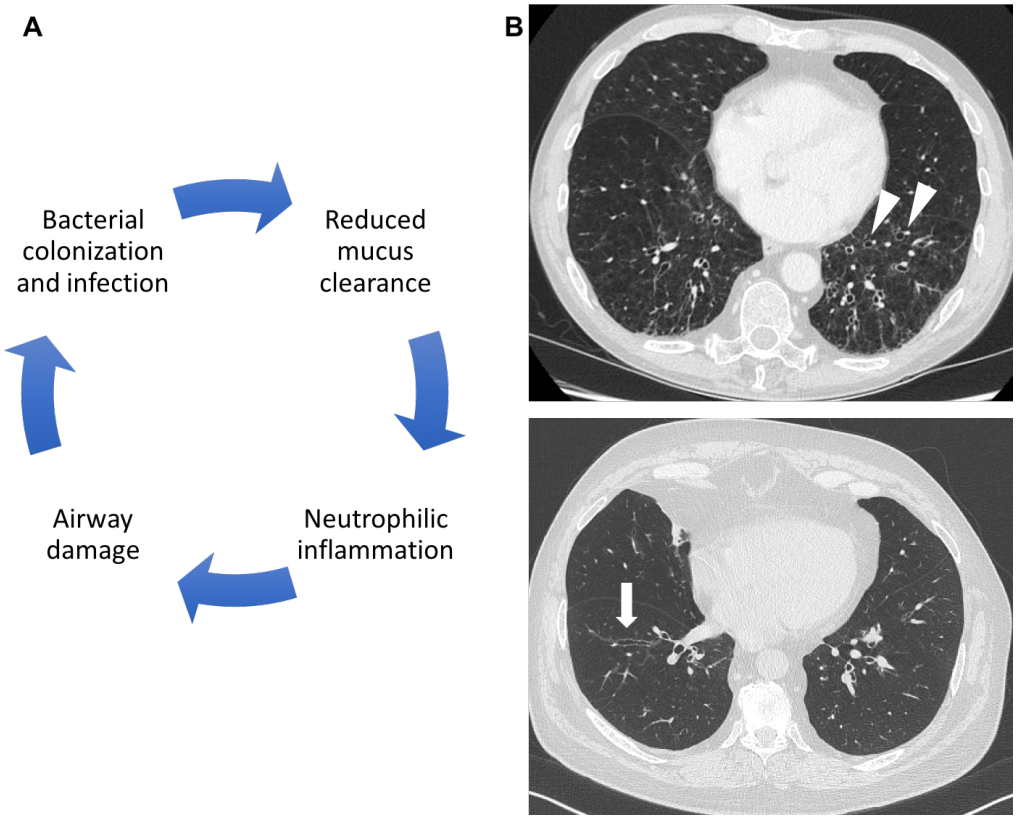
2.1 Pathogenesis and pathophysiology

Bronchiectasis is generally described as irreversible dilation of bronchial airways, accompanied by airway wall thickening and loss of distal narrowing. In 1986, Cole proposed a model to explain the pathogenesis of bronchiectasis (29). He hypothesized a vicious circle as the underlying mechanism, in which an infectious event results in reduced mucociliary clearance, facilitating persistent bacterial colonization, which in turn provokes chronic inflammation that causes structural damage to the airways (Figure 4A). Historically, three types of bronchiectasis were described based on their morphology: cylindrical, varicose and cystic, with the latter the most severe form. Thickening of bronchial airways is caused by inflammation, edema, ulceration, fibrosis and even micro-abscesses in later stages (30). The airway inflammation in bronchiectasis is driven by neutrophils, which are attracted by high concentrations of chemokines (31). These neutrophils exhibit a deficient phagocytosis (32) and release proteases. Neutrophil elastase plays a key role since it affects structural proteins, stimulates cytokine secretion, activates other proteinases and has toxic effects on cilia and cells (33). Next to neutrophils, also the number of macrophages is increased in bronchiectatic airways, although their role is not well defined (34). The role of adaptive immunity is complex since both immunodeficiency as hyperactive immunity may lead to bronchiectasis. The T helper type 17 pathway is likely to be involved, driving neutrophilia and mucus hypersecretion. Increased CD4+ and CD8+ T cells in the bronchial walls seem to be typical for bronchiectasis, but which of these are predominant and if they form ectopic lymphoid follicles differs between patients, possibly relating to the disease-initiating processes. (35) The most extensive study on the pathology of bronchiectasis dates back to the 50's, when Whitwell investigated 200 surgical lung specimens with bronchiectasis. He emphasized the role of small airways and suggested these lymphoid follicles in the bronchial walls of some patients as the cause of small airway obstruction (36).

Bacterial colonization is both a consequence and a driver of the disease. *Haemophilus influenzae* and *Pseudomonas aeruginosa* are the most commonly isolated potential pathogenic microorganisms (PPM) (37,38). Colonization with *Pseudomonas* species

represents a specific clinical phenotype (39) and is associated with exacerbation frequency, hospital admissions and worse quality of life (40,41). In recent years, differences in the lung microbiome of patients with bronchiectasis have been recognized, although interactions between microbiome and host immune response are not understood yet (38,42).

Figure 4: Pathogenesis and imaging of bronchiectasis



(A) Simplified version of the vicious circle underlying the pathogenesis of bronchiectasis. (B) HRCT images of lungs with bronchiectasis showing an increase in bronchoarterial ratio (arrowheads) and lack of tapering (arrow).

2.2 Clinical aspects

Next to the clinical presentation, diagnosis of bronchiectasis is based on imaging, with high-resolution computed tomography (HRCT) as the gold standard. The criteria to diagnose bronchodilation are based on descriptions by Naidich: a broncho-arterial ratio >1, lack of bronchial tapering or visible bronchus within 1cm of costal pleura or abutting the mediastinal pleura (43,44) (Figure 4B). Other possible features of bronchiectasis on HRCT are bronchial wall thickening, mucus plugging and mosaic attenuation of the parenchyma (45). However, there is no consensus on the minimal findings that have to be present on HRCT to label an airway as bronchiectatic and different studies use distinct criteria and definitions.

Progression and prognosis of the disease is variable, due to the clinical heterogeneity and the wide range of etiologies (39,46,47). Patients with bronchiectasis may suffer from exacerbations, recently defined as deterioration in three or more key symptoms (cough, sputum volume and/or consistency, sputum purulence, breathlessness and/or exercise tolerance, fatigue and/or malaise and haemoptysis) for at least 48h and requiring therapy change, determined by a clinician (48).

In terms of maintenance treatment, respiratory physiotherapy for airway clearance is essential. Long-term oral or inhaled antibiotics are appropriate in certain patients, next to treatment of exacerbations and eradication of *Pseudomonas Aeruginosa* at first isolation. Mucoactive and bronchodilator treatment may also be indicated for a subgroup of patients. (49)

2.3 Bronchiectasis and COPD

In addition to being chronic, inflammatory airway diseases, COPD and bronchiectasis have several characteristics in common. In both diseases, non-specific respiratory symptoms and exacerbations are present, inflammation is dominated by neutrophils and lung function may be obstructive. Nevertheless, they are fundamentally different in terms of diagnosis, therapeutic management and prognosis as described above for the individual conditions.

Although similarities between the two diseases may lead to misdiagnosis of COPD in patients with bronchiectasis or under-diagnosis of bronchiectasis in patients with COPD, the increased use of chest CT in clinical practice led to the determination of bronchiectasis in patients with COPD and the suggestion of a COPD-bronchiectasis overlap phenotype (50–52). However, the reported prevalence of bronchiectasis in COPD ranges widely, from 4% to 70%, due to differences in study subjects and radiological criteria (53). An association between COPD severity and bronchiectasis prevalence was a consistent finding in most studies (54,55). The other way around, COPD is also a frequently observed co-morbidity in bronchiectasis cohorts, present in 3-35% of patients (28,56,57).

Importantly, patients with COPD and bronchiectasis have a more severe course of disease with increased colonization by PPM (58,59), more exacerbations (58,60) and even higher mortality (59,61) based on several studies and confirmed in two recent meta-analyses (62,63). Moreover, the same trend is observed in studies that focus on bronchiectasis, where the

presence of COPD in subjects with bronchiectasis is reflected by worse disease and prognosis (57,64).

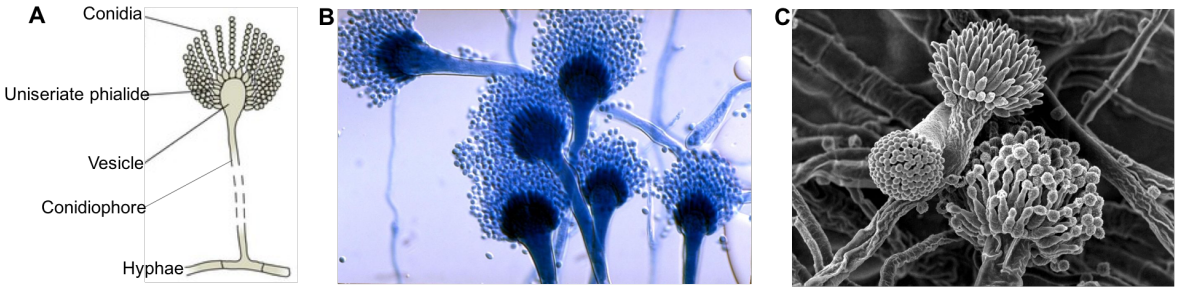
Next to this markedly higher morbidity in patients with COPD and bronchiectasis, the recognition of patients with both diseases is also relevant because of therapeutic implications. Both conditions are treated differently and the strategies applied in one are not always effective for -or may even worsen- the other. Whereas bronchodilation and inhaled corticosteroids (ICS) are among the cornerstones of COPD treatment (1), these therapies are less effective and, in case of ICS, even potentially harmful in patients with chronic infection, such as in bronchiectasis (65,66). On the other hand, inhalation of antibiotics and hypertonic substances, long-term macrolides, a long course of antibiotics for exacerbations and respiratory physiotherapy are essential in the management of bronchiectasis patients (49). So far, no clinical therapeutic trials were performed in patients with both COPD and bronchiectasis.

The clinical, therapeutic and prognostic implications of COPD-bronchiectasis overlap meet the definition of a clinical COPD phenotype (22). The appearance of bronchiectasis in COPD is mostly cylindrical, bilateral and bibasal, but the underlying mechanism for development of bronchiectasis in COPD is far from understood. If there is a causal relationship rather than that both diseases coincidentally exist in one patient, is not known. So far, no longitudinal studies are performed to demonstrate a causal relationship. However, with the vicious circle underlying bronchiectasis in mind (Figure 4A), it is plausible that patients with COPD enter the circle due to frequent exacerbations and chronic bronchial infection (53,62,63). Furthermore, some studies in bronchiectasis suggest an association between emphysema and bronchiectasis (67,68) and the number of bronchopulmonary segments with emphysema is significantly associated with clinical severity in post-infective and idiopathic bronchiectasis (69). In COPD, the sequence in which emphysema and bronchiectasis arise is unclear and underexplored, although the association between emphysema and bronchiectasis seems proportional in alpha-1 antitrypsin deficiency (70).

3. *Aspergillus fumigatus*

Aspergillus species are ubiquitous molds, belonging to the kingdom of fungi and with an important role in carbon and nitrogen recycling. They mainly inhabit soil and decaying vegetal material, although they are also present in the indoor environment. *Aspergillus fumigatus* (*A. fumigatus*) is the most common species of this genus to cause disease in animals and humans. The main difference compared to other common *Aspergillus* species is the ability to grow at 45°C. Furthermore, *A. fumigatus* has small, hydrophobic conidia that are easily dispersed into the air and inhaled on a daily basis (Figure 5). These conidia measure only 2 to 3µm, whereby they are capable to reach distal parts of the bronchial tree, all the way to the alveoli.

Figure 5: *Aspergillus fumigatus*



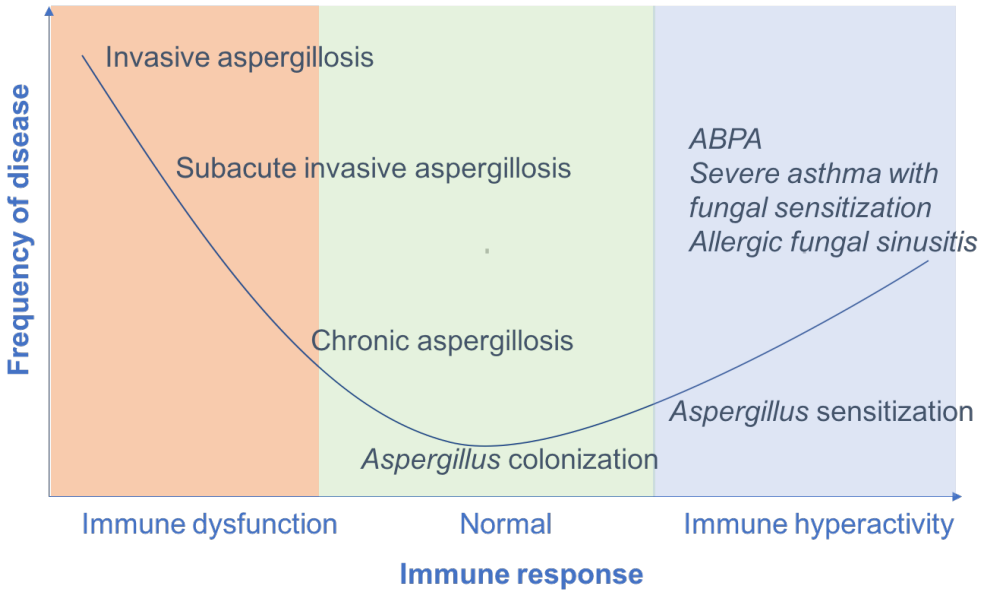
Aspergillus fumigatus morphology: (A) schematic (B) microscopic (C) electron microscopic.

3.1 *A. fumigatus* and respiratory disease

At least in healthy subjects, conidia are cleared from the airways by functional airway epithelium and by innate immune cells. The cornerstones of defense against *A. fumigatus* are mucociliary clearance, phagocytosis and the emergence of inflammatory pathways after ligation of fungal cell wall motifs to pathogen-recognition receptors on host cells (71,72). Alveolar macrophages phagocyte conidia and produce chemokines and cytokines if conidia germinate into hyphae. These inflammatory mediators attract cells, such as neutrophils, with the potential to kill hyphae, which are the tissue-invasive forms of the mold (73). Next to the innate immune response, adaptive immunity is activated after recognition of specific motifs on hyphae, stimulating helper T cells and regulatory T cells (72). The clinical spectrum of *Aspergillus*-associated disease is determined by host immunity and pulmonary

microenvironment, and ranges from asymptomatic colonization over hypersensitivity disorders to potentially lethal invasive disease (74) (Figure 6). Tissue invasion is uncommon and occurs mostly in the setting of neutropenia due to cytotoxic therapy for malignancies or immunosuppression with high doses of CS (71). Other risk factors for aspergillosis are massive exposure, damaged airway epithelium and inherited innate immune deficiency (75,76). In line with a disturbed lung microbiome, the mycobiome may as well undergo rearrangement in chronic respiratory disease (77), although our understanding of the human mycobiome is still in its infancy. In an immunocompetent host with chronic airway disease, *A. fumigatus* may remain and proliferate in the airways, due to reduced mucociliary clearance and/or disturbed antimicrobial functions. Fungi may innocently reside in the lumen, but may also be harmful by antigens and proteases (78), whereby worsening the already damaged status of the airway. In CF, a lung disease caused by mutations in the CFTR gene and characterized by bronchiectasis, colonization with *Aspergillus* is associated with worse disease (79,80). Also in asthma, both *Aspergillus* presence and sensitization are associated with reduced lung function (81–83). A major confounder in this field is the lack of sensitive, noninvasive methods to show *A. fumigatus* in the airway. Current studies are mainly based on fungal culture, which is not sensitive and not standardized (84).

Figure 6: Relation between host immune response and aspergillus disease



Graph showing relation between immune response (x-axis) and burden of aspergillus disease (y-axis). Adapted figure with permission (85).

3.2 *A. fumigatus* in COPD

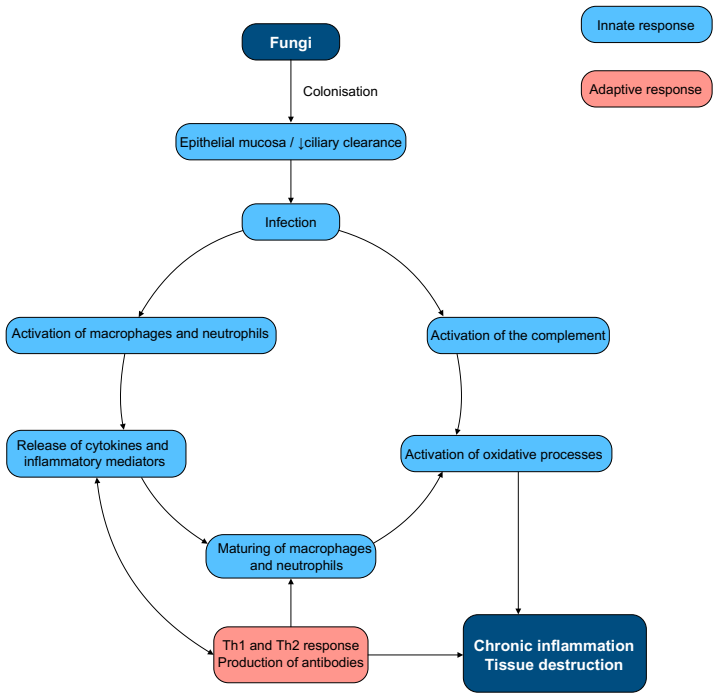
Patients with COPD have a twofold reason for being at risk for colonization by *Aspergillus* spp: reduced innate immunity by dysfunction of airway epithelial cells and alveolar macrophages on one hand and the use of inhaled or systemic CS affecting neutrophilic function on the other hand. Moreover, repeated courses of antibiotics may contribute to the risk (86). *A. fumigatus* is indeed commonly isolated in sputum samples of patients with COPD, but the few studies that were carried out, do not show an association with exacerbations (87–89). However, several retrospective studies showed that more than 20% of COPD patients with *A. fumigatus* detected in airway samples eventually developed aspergillosis (90,91). In patients diagnosed with invasive pulmonary aspergillosis without severe immune suppression, COPD is a frequent underlying disease (90,92–94), as it is in chronic pulmonary aspergillosis (75). These destructive and potentially life threatening aspergillus-associated diseases require anti-fungal treatment. Sensitization to *A. fumigatus*, meaning the presence of specific IgE against this mold, is associated with rhinosinusitis, asthma and allergic bronchopulmonary aspergillosis (ABPA) in patients with asthma and CF (Figure 6) (95). Whether sensitization to *A. fumigatus* is also meaningful in COPD has been rarely investigated, although an allergic COPD phenotype with more symptoms and exacerbations has been suggested (96). The rationale of therapeutic measures, such as antifungal treatment or reduction of CS use in colonized or sensitized patients is largely unknown, as both clinical and mechanistic research in this field is scarce.

3.3 *A. fumigatus* and bronchiectasis

As described above, patients with bronchiectasis are prone to airway colonization by micro-organisms due to reduced mucociliary clearance, dysfunctional innate immunity and structural modifications. Next to bacteria, also fungi such as *A. fumigatus* tend to remain in the airways (97). Interestingly, *A. fumigatus* may also have a causal role in the development of bronchiectasis (98). A well-known argument for this causal relation is ABPA. In this condition, mainly encountered in patients with asthma or CF, *Aspergillus* antigens provoke a hypersensitivity reaction with upregulation of T helper type 2 (Th2) inflammation. The inflamed bronchi eventually become bronchiectatic. The pathophysiology is incompletely understood, but both immune response and genetic susceptibility play a role (95). Although

research into a causal link is still limited, there is some indirect evidence from clinical and mechanistic studies of non-allergic pathways linking *Aspergillus* and bronchiectasis. On clinical grounds, colonization with *Aspergillus* conidia was associated with bronchiolitis obliterans syndrome, having bronchiectasis as a feature, after lung transplantation (99). Persistent isolation of *Aspergillus* species was associated with production of purulent sputum in bronchiectasis (97) and sensitization to *A. fumigatus* was associated with lung damage and bronchiectasis, irrespective of ABPA, in asthma (82,100). Underlying mechanisms may be the upregulation of mucus production and Th2 inflammation by *Aspergillus* proteases, which may contribute to development of bronchiectasis (101,102). Moreover, colonization by *A. fumigatus* hyphae caused a strong neutrophilic reaction in a murine model (103). Figure 7 represents how fungi may play a causal role in development and progression of bronchiectasis (104). In addition to these arguments, it is not unlikely that also genetic susceptibility and interactions with other microorganisms determine the risk to develop *A. fumigatus*-related bronchiectasis.

Figure 7: Suggested role of fungi in pathogenesis of bronchiectasis

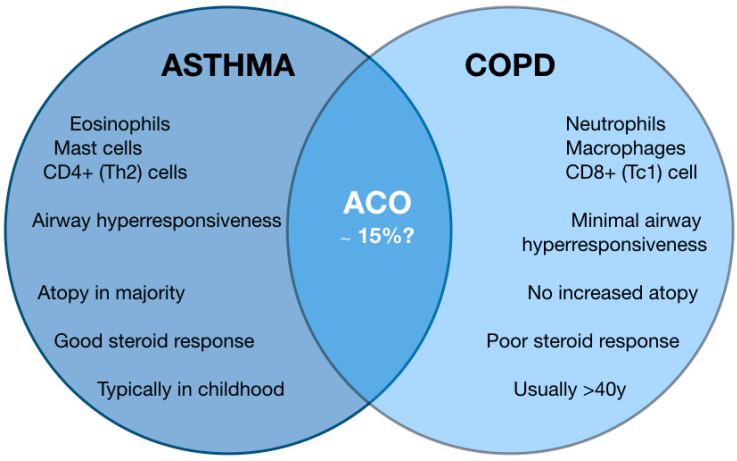


Proposed causal pathway between fungi and bronchiectasis. Figure based on (104) with permission.

4. Asthma-COPD overlap

Asthma is defined as "a heterogeneous disease, usually characterized by chronic airway inflammation and the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation" (105). The global prevalence of asthma is estimated at 5% (106). The overlap of COPD with asthma or asthma-COPD overlap (ACO), is a potential phenotype that gained a lot of interest last years. COPD and asthma are both prevalent and therefore it is not unlikely that they occur in the same individual, especially in subjects above 40 years of age with a history of considerable exposure to noxious stimuli. Although both are inflammatory, obstructive airway diseases, asthma and COPD generally have a different nature of inflammation, onset, course and treatment (Figure 8). Nevertheless, they may have clinical and physiological similarities (107). Asthma may progress to persistent airflow obstruction which is the hallmark of COPD (108,109). On the other hand, reversibility of airflow obstruction, which is characteristic for asthma, is often determined in COPD (110). Moreover, eosinophilic inflammation that was historically thought to be typical for asthma, is now proposed as a biomarker associated with exacerbations and better response to ICS in a subset of patients with COPD (111,112), whereas a neutrophilic phenotype is also recognized in asthma (113).

Figure 8: Schematic representation of overlap between asthma and COPD



Typical characteristics of asthma and COPD that may overlap between diseases. Figure reproduced with permission (114).

Different definitions and criteria for ACO have been proposed (Table 1), but the debate continues despite these efforts and many studies apply a self-created definition. The prevalence of ACO is highly dependent on the used definition and the studied population (115,116). In the general population, prevalence is estimated at 1.6-4.5% (115–117). In COPD and asthma populations, the prevalence of overlap varies tremendously, from below 10% to above 50% (116,118). What most reports have in common, is an age-dependent prevalence, with increased chance of having ACO at higher ages (119).

Table 1: Examples of proposed definitions and criteria for ACO

Global Initiative for Asthma (120)	ACO is characterized by persistent airflow limitation with several features usually associated with asthma and several features usually associated with COPD. ACO is therefore identified in clinical practice by the features that it shares with both COPD and asthma. This is not a definition, but a description for clinical use, as ACO includes several different clinical phenotypes and there are likely to be several different underlying mechanisms. Usual features of ACO: • Age ≥40 years, but may have had symptoms in childhood. • Persistent exertional dyspnea, although variability may be prominent. • Airflow limitation is not fully reversible, but often with current or historical variability. • Persistent airflow limitation. • History or doctor-diagnosed asthma, allergies, family history of asthma and/or history of noxious exposures. • Symptoms are reduced by treatment. Progression is usual and treatment needs are high. • Exacerbations may be more common than in COPD but are reduced by treatment. • Eosinophils and/or neutrophils in sputum.
European Respiratory Society round table consensus (121)	The committee recommends presence of all three major criteria and at least one minor criterion: <u>Major criteria</u> 1. Persistent airflow limitation in individuals ≥40 years. 2. ≥10 pack-years of tobacco smoking OR equivalent indoor or outdoor air pollution exposure. 3. Documented history of asthma before 40 years of age OR bronchodilator response of >400ml in FEV1. <u>Minor criteria</u> 1. Documented history of atopy or allergic rhinitis. 2. Bronchodilator response of FEV1 ≥200 ml and 12% from baseline values on ≥2 visits. 3. Peripheral blood eosinophil count of ≥300 cells/μL.
History-based (116,122)	Co-registration of asthma and COPD in the electronic record of the patient. OR Self-reported: history of both asthma and COPD reported by the patient.

4.1 Pathogenesis and pathophysiology

Airway obstruction in asthma mainly results from smooth-muscle spasm, next to mucus production and inflammatory infiltrates. Inflammation is predominantly eosinophilic and dependent on Th2 lymphocytes, although inflammation in asthma is heterogeneous and may also be neutrophilic, mixed or paucigranulocytic in origin. Host-environment interactions play an important role in the pathogenesis (113).

The pathophysiology of COPD was described above. Despite the inflammatory differences, the profile of mediators in sputum and serum does not permit to distinguish between asthma, COPD and ACO (123,124). Moreover, asthma and COPD have common risk factors, such as bronchial hyperreactivity (125–127), gene expression profile (128–130), atopy (113,131,132) and early life events (133,134). Furthermore, asthma itself is a risk factor for COPD (135). This complex combination of differences and similarities between the diseases results in an ongoing debate about the pathophysiological link between asthma and COPD for almost 60 years now. Whereas Orie and colleagues hypothesized that chronic airway diseases have one common origin with different clinical and phenotypic expressions due to endogenous and exogenous factors (Dutch hypothesis) (136,137), others are in favor of a completely different underlying mechanism in both diseases (British hypothesis) (138,139). The discussions and uncertainties continue to date, since pros and cons have been discovered for both opinions.

4.2 Clinical aspects

Respiratory symptoms in ACO are similar to asthma and COPD symptoms; both exertional dyspnea (as in COPD) and variability in symptoms (as in asthma) may be expected (120). Because of the aspecificity, few studies investigated differences in respiratory symptoms between ACO and COPD. So far, no criteria or tests are validated for the diagnosis of ACO. Moreover, no ACO-specific biomarker has been discovered. In fact, ACO is an umbrella term that includes smoking asthmatics with persistent airflow limitation, and patients with COPD that have increased blood eosinophil counts (140), which makes it hard to find a golden standard for diagnosis.

Contrasting results in terms of morbidity, progression and mortality are reported in patients with ACO versus COPD. Regarding exacerbations, most studies show more frequent and severe exacerbations (117,119,129,141–143), although others show the opposite (116,144,145). Also with regard to FEV1 decline, opposite results are found in the current literature; from less (146) over equal (143,145) to more pronounced (142,147) decline in ACO. Only one study could show higher mortality in ACO compared to COPD (142), which is remarkable since lower mortality in ACO (144,148) contrasts with the finding that COPD patients with airway hyper-responsiveness have a higher mortality (149,150). The reason for these conflicting results is probably the difference in severity of baseline airway obstruction and the difference in definition used for ACO, illustrating the divergent strategies applied in this field.

The main reason for distinguishing ACO from COPD and asthma is the therapeutic implication. Smoking cessation is evident, as is avoidance of relevant allergen exposure. Unfortunately, hardly any randomized controlled trial has been performed to investigate pharmaceutical therapy in ACO, since asthma patients who smoked are generally excluded from asthma trials and COPD patients with a history of asthma are generally not included in COPD trials. Due to infectious risks (65,151), ICS are only reserved for a subset of patients with COPD (1,111,152). In ACO on the other hand, ICS are considered essential because of their valuable role in the management of asthma. A beneficial effect of ICS in ACO has been shown in some small studies (153–155), but caution is necessary since risks and benefits of ICS in ACO have not yet been weighed. Next to ICS, recognition of ACO may lower the threshold to prescribe tiotropium (156,157) and azithromycin (158,159) in former asthma, and montelukast in former COPD. Even more challenging is the opportunity of treating this patients with the biologicals that are currently used in severe asthma. Benefits and safety of these molecules, such as omalizumab (anti-IgE) and mepolizumab (anti-IL5), in ACO will hopefully be revealed from future, prospectively designed studies.

If we want to offer personalized and evidence based treatment to our patients with ACO, we urgently need a consistent approach for diagnosis, taking extensive pheno- and endotyping into account. Only this will enable us to conduct well-designed studies that include "real-world" patients to discover potential biomarkers and investigate efficacy of therapy in ACO.

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Chapter 2

Rationale and objectives

General rationale

As elaborated in the introduction, the understanding of phenotypes, endotypes, biomarkers and treatable traits within COPD will result in more precise management strategies in our patients.

We explored some recently introduced and challenging phenotypes of COPD.

Aspergillus fumigatus and bronchiectasis in COPD (Chapter 3)

Both *A. fumigatus* colonization and sensitization associate with unfavorable outcomes and bronchiectasis in asthma and CF. Some indirect evidence from clinical and mechanistic studies suggest a causal role in development of bronchiectasis. These associations have never been investigated in COPD. A major limitation in this field is the lack of sensitive, non-invasive detection methods to uncover the presence of this fungus in the airways.

Does an association between A. fumigatus sensitization and bronchiectasis exist in COPD?

In chapter 3A we aimed to investigate sensitization to *A. fumigatus* in COPD versus controls and more specifically explore an association with bronchiectasis. An additional objective was to evaluate the role of sensitization to recombinant *A. fumigatus* antigens.

Do patients with COPD and bronchiectasis have more A. fumigatus in their airways? Is there a better way to detect and prevent fungal presence in these patients?

In chapter 3B the main aim was to demonstrate a more abundant presence of *A. fumigatus* in airways of patients with COPD-bronchiectasis overlap compared to COPD. In order to achieve this objective, the performance and feasibility of *A. fumigatus* culture, sputum galactomannan and *A. fumigatus* PCR were investigated. Finally, this chapter evaluated CS as a potential risk factor for presence of *A. fumigatus* in sputum samples.

Airway morphometry of COPD lungs with bronchiectasis (Chapter 4)

Although bronchiectasis gained a lot of interest as a research topic, there are no advanced imaging studies in bronchiectasis that focus on the airway generations beyond the resolution of conventional CT. Moreover, the understanding of the pathophysiological processes underlying COPD-bronchiectasis overlap is very limited.

How and where does the composition of the bronchial tree differ in COPD patients with bronchiectasis?

In this chapter, we focused on alterations in the bronchial tree of COPD lungs with bronchiectasis compared to COPD lungs without bronchiectasis and unused donor lungs. We aimed to study numbers and diameters of airways throughout the entire bronchial tree by the use of high resolution CT of explanted lungs, microCT of lobes and microCT of tissue cores.

Asthma-COPD Overlap (Chapter 5)

A correct diagnosis of obstructive airway disease is necessary, as disease progression and management differ between asthma, COPD and ACO. Nevertheless, current guidelines contribute insufficiently to differential diagnosis, which impedes a personalized and evidence-based approach.

How can we consistently differentiate ACO from asthma and COPD? Which tests are essential in doing so?

Chapter 5 includes a prospective study, in which we aimed to test the accuracy of a diagnostic algorithm for obstructive airway diseases. With the help of machine learning, we also evaluated which tests contributed most to the diagnosis.

Chapter 3

Aspergillus fumigatus and bronchiectasis in COPD

A. Sensitization to *Aspergillus fumigatus* as a risk factor for bronchiectasis in COPD

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ABSTRACT

Background

Bronchiectasis-COPD overlap presents a possible clinical phenotype of COPD, but it is unclear why it develops in a subset of patients. We hypothesized that sensitization to *Aspergillus fumigatus (A. fumigatus)* associates with bronchiectasis in COPD and occurs more frequently in vitamin D deficient patients.

Methods

This observational study investigated sensitization to *A. fumigatus* in an outpatient clinical cohort of 300 COPD patients and 50 (ex-) smoking controls. Total IgE, *A. fumigatus* specific IgE against the crude extract, against the recombinant antigens and *A. fumigatus* IgG were measured by ImmunoCAP fluorenzyme-immunoassay. Vitamin D was measured by radioimmunoassay and computed tomography images of the lungs were scored with the modified Reiff score.

Results

Sensitization to *A. fumigatus* occurred in 18% of COPD patients compared to 4% in controls (p=0.0110). In all, 31 COPD patients (10%) were sensitized to the crude extract and 24 patients (8%) had only IgE against recombinant antigens. *A. fumigatus* IgG levels were significantly higher in the COPD group (p=0.0473). Within COPD, *A. fumigatus* sensitized patients were more often male (p=0.0293) and had more often bronchiectasis (p=0.0297). *Pseudomonas aeruginosa* and *Serratia marcescens* were more prevalent in historical sputum samples of sensitized compared to non-sensitized COPD patients (p=0.0436). Vitamin D levels were comparable (p=0.2057). Multivariate analysis demonstrated that sensitization to recombinant f1 or f3 had a 2.8 fold increased risk for bronchiectasis (p=0.0030).

Conclusions

These results highlight a potential role for sensitization to *A. fumigatus* in COPD-related bronchiectasis.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is highly prevalent and a leading cause of morbidity and mortality worldwide. Both social and economic burden continue to rise (1). It has become widely accepted that the degree of airflow limitation is insufficient to estimate the severity and heterogeneity of COPD. Symptoms, exacerbations and comorbidities are well-established determinants (2). Furthermore, there is growing interest in phenotyping the disease not only based on clinical characteristics but also on genetic, molecular and cellular parameters (3,4). By differentiating phenotypes, a more personalised and effective therapy might be achieved for subgroups of patients.

Jamieson et al. demonstrated that COPD patients with allergic sensitization experience more symptoms and exacerbations (5). Although hypersensitivity to *Aspergillus fumigatus (A. fumigatus)* was not measured here, two other studies have reported on the higher prevalence of *A. fumigatus* sensitization in COPD and its association with worse lung function (6,7). Sensitization is defined by the presence of allergen-specific IgE, which is routinely measured by skin prick test or in vitro immunoassay. In general, a crude extract of *A. fumigatus* is used, although purified or recombinant antigens of *A. fumigatus* are more specific (8). Different studies suggest a role for these recombinant antigens in distinguishing patients with and without allergic bronchopulmonary aspergillosis (ABPA) in cystic fibrosis (CF) and asthma (9,10). Whether *A. fumigatus* recombinant antigens are also of interest in other lung diseases such as COPD, is not known.

COPD-bronchiectasis overlap syndrome is a potential clinical phenotype (11). Prevalence varies greatly depending on the definition and methodology. Recent literature shows that bronchiectasis is clinically important (12–14) and compose an independent risk factor for mortality in COPD (14–16). Moreover, diagnose bronchiectasis in COPD has an impact on therapeutic considerations such as the safety of inhaled corticosteroids, the need for inhaled antibiotics or maintenance therapy with azithromycin and the antimicrobial management of exacerbations. At present, the pathogenesis of non-CF bronchiectasis is explained by a vicious circle in which inflammation causes structural damage and impaired mucus clearance, which in turn promotes bacterial colonization and infection, again resulting in more inflammation (17). The triggers for the development of bronchiectasis in COPD are not

known. Given the multiple links between *Aspergillus species* and bronchiectasis (18), we hypothesize that *A. fumigatus* may play an important role in the development of COPD-related bronchiectasis. As *A. fumigatus* sensitization and bronchiectasis may have a common link through suppression of the vitamin D signaling pathway (19–21), we also speculate that vitamin D deficiency, which is frequently observed in COPD and bronchiectasis (22,23), may also contribute to *A. fumigatus* sensitization.

We performed an observational study in a COPD cohort and a smoking control group recruited from the outpatient clinic of the University Hospitals of Leuven. We explored if *A. fumigatus* sensitization was more prevalent in COPD patients versus controls by measuring IgE against the crude *A. fumigatus* extract and 5 commercially available *A. fumigatus* recombinant antigens. We hypothesized that *A. fumigatus* sensitized COPD patients have different clinical characteristics, lower vitamin D serum levels and more bronchiectasis compared to COPD patients without *A. fumigatus* sensitization.

METHODS

Study design and subjects

In this observational study, subjects were retrospectively selected from the Leuven COPD cohort, a DNA-, plasma- and serumbank of a clinical based COPD population and controls (NCT00858520). Inclusion criteria were an age of ≥50 years and smoking history of ≥10 pack-years. A recent diagnosis of cancer, respiratory disorders other than COPD, major thoracic surgery and solid organ transplantation were exclusion criteria. Medical history, clinical parameters, therapy and the modified Medical Research Council breathlessness scale (mMRC)(24) were surveyed. Exacerbations were defined as acute events with worsening of respiratory symptoms that was beyond normal day-to-day variations and led to a change in medication (2). The number of exacerbations in the year prior to inclusion was recorded. The combined assessment as proposed by The Global Initiative for Chronic Obstructive Lung Disease (GOLD) was used to stage the severity of COPD: breathlessness (mMRC) and exacerbation history were used to subdivide COPD patients into 4 stages; A, B, C and D (2). The study was approved by the local Ethics Committee (Medical Ethical Board of the University Hospitals Leuven, Belgium - ML11081) and all patients provided written informed consent. We selected 300 patients with an established diagnosis of COPD based on a post-bronchodilator FEV1/FVC ratio <0.7, who were included between October 2007 and September 2013. Selection of our study patients was based on Caucasian race, the availability of complete pulmonary function data and 1mm thin-sliced computed tomography (CT) imaging of the thorax. The control group consisted of 50 (ex-) smokers with a post-bronchodilator FEV1/FVC ratio >0.7, who were enrolled in a lung cancer screening trial (NELSON, ISRCTN63545820) between October 2007 and October 2009.(25)

Pulmonary function

Post-bronchodilator spirometry was measured at the time of recruitment using standardized equipment (Sensormedics Whole Body Plethysmograph, Viasys Healthcare, Vilvoorde, Belgium), according to the American Thoracic Society/European Respiratory Society guidelines (26). Diffusion capacity was measured by the single-breath carbon monoxide gas transfer method (27). Results are reported as percentages predicted of

reference values. COPD was diagnosed based on a post-bronchodilator FEV1/FVC ratio of <0.7 and the post-bronchodilator FEV1 was used to classify patients according to the GOLD classification (2).

CT images

All subjects had a CT of the thorax within 1 year of enrollment. CT scans were taken for a variety of indications, which resulted in the use of different protocols in the COPD group. Nevertheless, only patients with slices of 1mm were included in this study. Subjects in the control group were all scanned according to the same protocol as described before (25). Airway dilatation was determined based on Naidich's descriptions: broncho-arterial ratio >1, lack of tapering, presence of bronchus within 1cm of costal pleura or abutting the mediastinal pleura (28). All scans were blinded to the other data and scored for bronchiectasis using the modified Reiff score. This score ranges from 0 to 18 by assessing the number of involved lobes (the lingula considered separately) and the degree of bronchodilation (1=tubular, 2=varicose and 3=cystic) (29). A score of ≥2 was considered clinical relevant because minor bronchial dilatation is also described in healthy individuals (30).

Blood analysis

Plasma was collected in cryotubes and stored at -80°C. Total IgE, IgE against *A. fumigatus* extract, against *A. fumigatus* recombinant antigens f1-f4, f6 and *A. fumigatus* IgG were determined by ImmunoCAP fluoroenzyme-immunoassay using an ImmunoCAP 1000 instrument (Phadia AB, Uppsala, Sweden). A cut-off of 114kU/L was used for total IgE (31) and specific IgE values >0.35kU/L were considered positive. We present the specific IgE results as proportions rather than absolute values due to the detection limit and the clinical relevance. 25-hydroxy-vitamin D was measured using radioimmunoassay (RIA, DiaSorin, Brussels, Belgium) with results expressed in micrograms per liter.

Sputum

In the COPD group, the presence of potential pathogenic microorganisms (PPM) in historical sputum cultures was assessed from laboratory reports. All sputum cultures performed in our hospital since 2002 were evaluated. These sputum samples were mainly

collected during bronchitis or exacerbation episodes. We registered if they ever had PPM cultured and if so, which different species were found. All cultures were performed according to the standard protocol of our hospital. In brief, 10µL of sputum sample was inoculated on 4 different agars; blood agar, Haemophilus specific agar, mannitol salt agar and MacConkey agar. These were cultured for 2 days.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 4 (GraphPad Software, La Jolla, CA USA) and SAS software version 9.4 (SAS Institute, Cary, NC USA). Normality was tested by the Shapiro-Wilk test; none of the continuous variables were normally distributed. Univariate comparisons between groups were performed by Wilcoxon rank-sum test and presented as median ± interquartile range. Proportions of discrete variables were compared with χ^2 test and presented as absolute numbers and percentages. We built a multivariate logistic regression model to study the association between sensitization to rAsp f1 or rAsp f3 and bronchiectasis in COPD. After performing bivariable logistic regression models with modified Reiff score ≥2 as exposure, potential confounders of the association between sensitization and the presence of bronchiectasis were included in the final model if they (1) changed the estimate of the multivariable model ≥10% or (2) the variable was significantly associated with the presence of bronchiectasis. p values <0.05 were considered significant in all analyses.

RESULTS

Study group characteristics

350 subjects were included in this study; 300 patients with COPD and 50 (ex-) smoking controls. There was no difference in gender. Patients in the COPD group were older, smoked more and had a lower mean BMI than controls (Table 1). The COPD group had a median FEV1 of 43% predicted and a median diffusion capacity of 46% predicted. The majority of COPD patients were staged in GOLD class 2 and 3, or groups B and D (32). In vitamin D non-supplemented COPD patients (n=240) a significantly lower median vitamin D level was observed compared to the control group (17.3 versus 28.5 µg/L, p<0.0001). The proportion of subjects with bronchiectasis was higher in COPD compared to controls (33% versus 10%, p=0.0010). More detailed information about the study groups is shown in Table 1.

Table 1: Study group characteristics

Characteristic	Control	COPD	P-values
Number	50	300	
Men (%)	37 (74.0)	215 (71.7)	0.7337
Age, years	62 (58-68)	67 (61-74)	**0.0002**
Pack-years	35.5 (26.8-46.8)	48.9 (34.0-64.1)	**0.0001**
Current smokers (%)	26 (52)	125 (42)	0.1781
BMI, kg/m²	27.3 (23.9-29.4)	24.1 (20.7-27.5)	**0.0004**
FEV1, L	2.9 (2.4-3.4)	1.2 (0.9-1.6)	NA
FEV1, % pred	104 (92-111)	43 (34-58)	NA
DLCO, mmol/min/Kpa	7.5 (6.1-8.9)	3.8 (3.0-5.0)	NA
DLCO, % pred	83 (78-94)	46 (36-58)	NA
GOLD 1 / 2 / 3 / 4, %	NA	5.7 / 32.0 / 45.7 / 16.7	NA
GOLD assessment A / B / C / D, %	NA	4.0 / 21.3 / 5.3 / 69.3	NA
Vitamin D, µg/L	28.5 (21.2-35.5)	17.3 (12.4-23.1) [a]	**<0.0001**
Modified Reiff score ≥2 (%)	5 (10)	99 (33)	**0.0010**

Data are presented as n, n (%), median (interquartile range) or %. BMI: body mass index, FEV1: forced expiratory volume in 1 second, % pred: percentage predicted, DLCO: diffusion capacity of the lung for carbon monoxide, GOLD: Global initiative for chronic Obstructive Lung Disease, NA: not applicable. [a] based on 240 COPD patients after exclusion of subjects taking vitamin D supplements. P-values <0.05 are captured in bold.

Total IgE and Aspergillus fumigatus specific antibodies

Total IgE, *A. fumigatus* specific IgE against the crude extract, the recombinants, and *A. fumigatus* specific IgG were measured to determine antibody production against *A. fumigatus*. Details of these results are given in Table 2. There was a tendency to higher total IgE levels and more prevalent IgE against *A. fumigatus* extract in COPD patients compared to controls (Figure 1A,B). If recombinant antigens were considered, significantly more COPD patients than controls had IgE against rAsp f1 (12% versus 2%, p=0.0332) (Figure 1B). Sensitisation to *A. fumigatus* extract and to rAsp f1 occurred in different patients: out of 50 patients sensitized to one of these two allergens, only 17 patients developed IgE antibodies >0,35kU/L against both *A. fumigatus* extract and rAsp f1 (Table 3). IgEs against rAsp f2, rAsp f4 and rAsp f6 were only rarely present in both groups, while there was no significant difference in IgE against rAsp f3 (Figure 1B). The difference in sensitization to at least one of the tested allergens was statistically significant: 55 COPD patients (18.3%) versus 2 controls (4%), p=0.0110 (Figure 1B). Furthermore, COPD patients had a higher median level of *A. fumigatus* IgG (31 mg/L versus 20.7 mg/L, p=0.0473) (Figure 1C).

Table 2: Total IgE and *A. fumigatus* specific antibodies

Variable	Control	COPD	P-values
Number	50	300	
Total IgE, kU/L	42.5 (16.5-105.5)	52.0 (17.0-198.5)	0.3068
Total IgE >114 kU/L (%)	11 (22)	103 (34.3)	0.0849
A. fumigatus extract IgE (%)	1 (2)	31 (10.3)	0.0584
rAsp f1 IgE (%)	1 (2)	36 (12)	**0.0332**
rAsp f2 IgE (%)	1 (2)	7 (2)	0.8839
rAsp f3 IgE (%)	1 (2)	20 (6.7)	0.1983
rAsp f4 IgE (%)	0 (0)	3 (1)	0.4776
rAsp f6 IgE (%)	0 (0)	4 (1.3)	0.4115
Sensitized in total (%)[a]	2 (4)	55 (18.3)	**0.0110**
A. fumigatus IgG, mg/L	20.7 (9.6-42.8)	31.0 (15.9-48.5)	**0.0473**

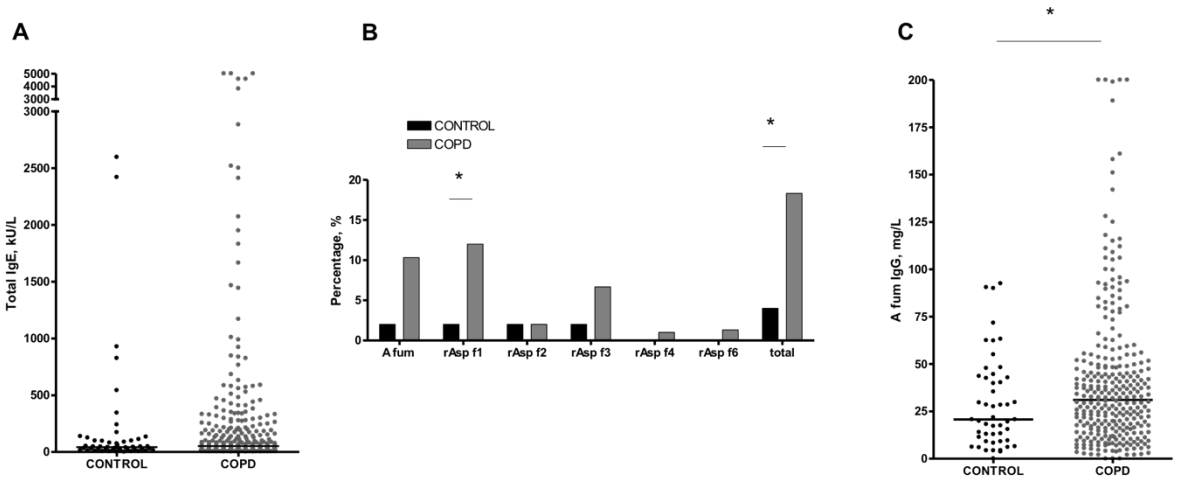
Results are presented as n, median (interquartile range) or n (%). *A. fumigatus: Aspergillus fumigatus*. rAsp f: recombinant of *Aspergillus fumigatus*. [a]Sensitization to at least one of the *A. fumigatus* allergens including the extract. P-values <0.05 are captured in bold.

Table 3: IgE against *A. fumigatus* extract and rAsp f1 in a contingency table

	rAsp f1 IgE present	rAsp f1 IgE absent	Row total
A. fumigatus **extract IgE present**	17 (5.7)	14 (4.7)	31 (10.3)
A. fumigatus **extract IgE absent**	19 (6.3)	250 (83.3)	269 (89.7)
Column total	36 (12.0)	264 (88.0)	300 (100)

Data are presented as n (%). *A. fumigatus* extract: *Aspergillus fumigatus* extract. rAsp f1: recombinant 1 of *Aspergillus fumigatus*.

Figure 1: Total IgE and *A. fumigatus* specific antibodies in patients with COPD versus controls



Antibody levels were measured in plasma by ImmunoCAP fluorenzyme-immunoassay using an ImmunoCAP 1000 instrument (Phadia AB, Uppsala, Sweden). a) Median values of total IgE. No significant difference between controls and COPD, p=0.3068. b) Proportions of subjects with IgE against different *Aspergillus fumigatus (A. fumigatus)* allergens: extract of *A. fumigatus* (*A. fumigatus*), *A. fumigatus* recombinant f1 (rAsp f1), *A. fumigatus* recombinant f2 (r Asp f2), *A. fumigatus* recombinant f3 (rAsp f3), *A. fumigatus* recombinant f4 (rAsp f4) and *A. fumigatus* recombinant f6 (rAsp f6). Total shows the proportion of participants sensitized against at least one of the tested allergens. * p<0.05 c) Median values of *Aspergillus fumigatus (A. fumigatus)* specific IgG. Difference between COPD and control was significant, * p=0.0473.

Differences between sensitized and non-sensitized COPD patients

Clinical and demographic characteristics were compared between 55 *A. fumigatus* sensitized and 245 *A. fumigatus* non-sensitized COPD patients. In the sensitized group, the proportion of men was significantly higher (83.6% versus 69%, p=0.0293). There was no difference in age, BMI, smoking history and use of inhaled corticosteroids. Furthermore, lung functional variables and GOLD stages were not different and sensitized patients did not experience more exacerbations compared to non-sensitized patients. Vitamin D levels were

comparable in the sensitized versus non-sensitized COPD patients (16.2 versus 18.1 ng/ml, p=0.2057). However, median values of specific *A. fumigatus* IgG were higher in *A. fumigatus* sensitized patients (43.2 mg/L versus 27.7 mg/L, p<0.0001), who also had significantly more bronchiectasis compared to non-sensitized patients (45.5% versus 30.2%, p=0.0297). Data are presented in Table 4.

Table 4: *A. fumigatus* sensitized versus non-sensitized COPD patients

Characteristic	COPD non-sensitized to *A. fumigatus*	COPD sensitized to *A. fumigatus*	P-values
Number (%)	245 (81.7)	55 (18.3)	
Men (%)	169 (69.0)	46 (83.6)	**0.0293**
Age, years	67 (61-74)	68 (64-74)	0.2767
BMI, kg/m²	24.4 (20.9-27.7)	23.3 (19.7-26.9)	0.0785
Pack-years	48.0 (33.5-63.8)	52.5 (35.3-66.0)	0.3518
Current smoking (%)	99 (40.6)	26 (47.3)	0.3629
ICS (%)	197 (80.4)	49 (89.1)	0.1299
FEV1, L	1.18 (0.86-3.93)	1.70 (0.83-2.86)	0.7632
FEV1, % pred	43 (34-58)	41 (33-56)	0.4461
DLCO, mmol/min/Kpa	3.9 (3.0-9.6)	3.6 (2.7-7.7)	0.4038
DLCO, % pred	47(37-59)	42 (32-58)	0.1618
GOLD 1 / 2 / 3 / 4, %	5.7 / 33.1 / 44.5 / 16.7	5.5 / 27.3 / 50.9 / 16.4	0.8291
GOLD assessment A / B / C / D, %	3.7 / 22.5 / 4.9 / 69.0	5.5 / 16.4 / 7.3 / 70.9	0.6515
≥2 exacerbations/year (%)	102 (41.6)	28 (50.9)	0.2096
Vitamin D, µg/L [a]	18.1 (12.4-24.8)	16.2 (12.4-21.2)	0.2057
Vitamin D ≤ 20 µg/L (%) [a]	112 (56.9)	30 (69.8)	0.1185
A. fumigatus IgG, mg/L	27.7 (13.9-44.4)	43.2 (27.0-66.0)	**<0.0001**
Modified Reiff score ≥2 (%)	74 (30.2)	25 (45.5)	**0.0297**
Sputum PPM (%) [b]	94 (59.5)	30 (76.9)	**0.0436**

Data are presented as n (%), median (interquartile range) or %. *A. fumigatus: Aspergillus fumigatus*. BMI: body mass index, ICS: inhaled corticosteroids, FEV1: forced expiratory volume in 1 second, % pred: percentage predicted, DLCO: diffusion capacity of the lung for carbon monoxide, GOLD: Global initiative for chronic Obstructive Lung Disease, PPM: potential pathogenic micro-organisms, [a] based on 240 COPD patients after exclusion of subjects taking vitamin D supplements; 197 non-sensitized and 43 sensitized patients. [b] based on 197 patients of which sputum results were available; 158 non-sensitized and 39 sensitized patients. P-values <0.05 are captured in bold.

Interestingly, bronchiectasis was only detected in patients with sensitization to recombinants. None of the 9 patients who were only sensitized to the crude extract -and therefore not to any of the recombinant allergens- had bronchiectasis. In contrast, patients with IgE against rAsp f1, and especially against rAsp f3, had significant more bronchiectasis than patients without sensitization to these allergens (Table 5). Results of sputum cultures were available in 197 patients. The median number of sputum samples per patient was not different between

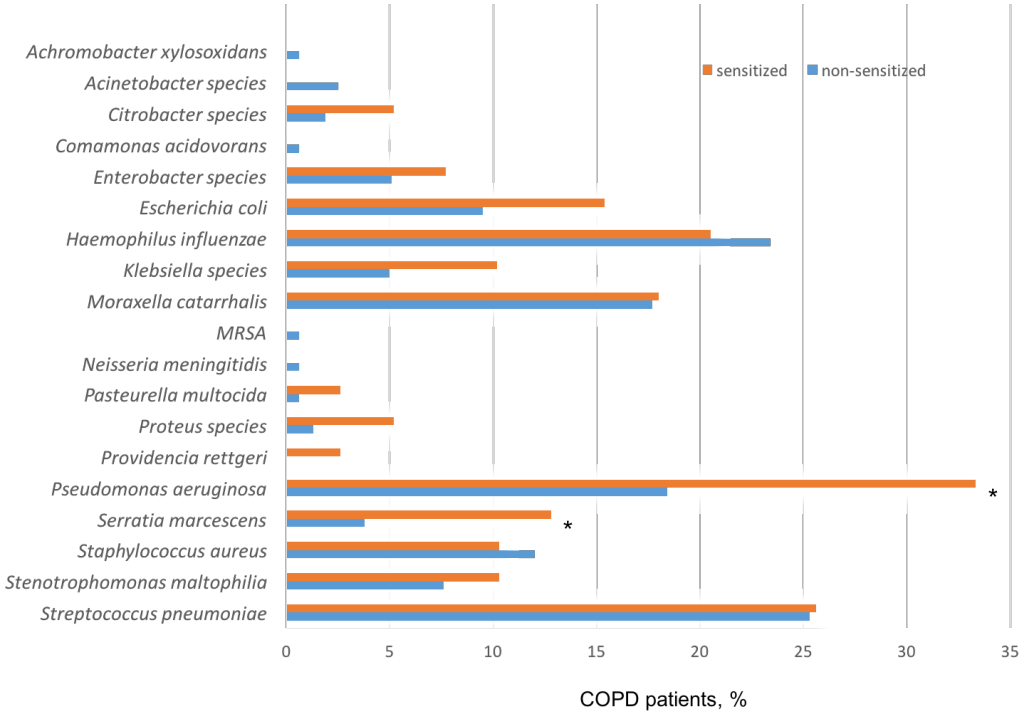
groups (4 (IQR 2-6) in sensitized patients and 3 (IQR 1-5) in non-sensitized patients, p=0.0692). PPM were significantly more prevalent in *A. fumigatus* sensitized versus non-sensitized patients (76.9% versus 59.5%, p=0.0436) (Table 4). Sputum cultures showed no difference in presence of typical COPD pathogens like *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catharralis*. In contrast, *Pseudomonas aeruginosa* and *Serratia marcescens* were more common in sputum cultures of *A. fumigatus* sensitized patients (Figure 2).

Table 5: *A. fumigatus* sensitization and bronchiectasis in COPD

Variables	COPD patients (n)	Modified Reiff score ≥2 (%)	P-values
IgE *A. fumigatus* extract	31	12 (38.7)	0.4752
IgE *A. fumigatus* extract OR rAsp f1-4, f6	55	25 (45.5)	**0.0297**
IgE *A. fumigatus* extract without recombinants	9	0 (0)	
IgE rAsp f1	36	18 (50)	**0.0208**
IgE rAsp f3	20	12 (60)	**0.0079**

Data are presented as n, n (%). *A. fumigatus* extract: *Aspergillus fumigatus* extract. rAsp f: recombinant of *Aspergillus fumigatus*. P-values <0.05 are captured in bold.

Figure 2: Potential pathogenic microorganisms present in sputum samples of COPD patients by *A. fumigatus* sensitization status.



Occurrence of different species in sputum cultures. MRSA: methicillin-resistant *Staphylococcus aureus*, *p<0.05.

Multivariate analysis with logistic regression showed a significant association between bronchiectasis, sensitization to rAsp f1 or rAsp f3 (p=0.0030) and age in COPD, independent of gender, BMI, diffusion capacity and presence of ≥2 exacerbations/year (Table 6). The significant association between rAsp f1 or rAsp f3 sensitization and bronchiectasis was confirmed with the same model in the subgroup of patients with sputum samples (n=197, p=0.0021), additionally corrected for the presence of PPM (Table 6).

Table 6: Association of bronchiectasis (modified Reiff ≥2) and sensitization to rAsp f1 or rAsp f3 in COPD by a logistic regression model

Variables	In all COPD patients (n=300)		In COPD patients with sputum samples (n=197)	
	OR (95% CI)	P-values	OR (95% CI)	P-values
Sensitization to rAsp f1 or rAsp f3	2.8 (1.42 – 5.53)	**0.0030**	3.4 (1.56 – 7.48)	**0.0021**
Gender	1.5 (0.81 – 2.75)	0.1953	1.8 (0.82 – 4.04)	0.1424
Age	1.0 (1.00 – 1.07)	**0.0349**	1.03 (0.99 – 1.07)	0.2064
BMI	1.0 (0.91 – 1.01)	0.1198	0.96 (0.89 – 1.03)	0.2155
DLCO, % pred	1.0 (0.97 – 1.00)	0.1353	0.98 (0.96 – 1.01)	0.1572
≥2 exacerbations/year	1.6 (0.97 – 2.72)	0.0642	2.11 (1.13 – 3.94)	**0.0188**
PPM in sputum	NA		0.68 (0.35 – 1.32)	0.2573

OR: odds ratio, CI: confidence interval. BMI: body mass index, DLCO: diffusion capacity of the lung for carbon monoxide, % pred: percentage predicted, PPM: potential pathogenic micro-organisms, NA: not applicable. P-values <0.05 are captured in bold.

DISCUSSION

This study shows that sensitization to *A. fumigatus* occurs more frequently in COPD patients compared to smoking controls, particularly when rAsp f1 is taken into account. *A. fumigatus* IgG levels are also significantly higher in the COPD group. *A. fumigatus* sensitized COPD patients are more often male, with more proteobacteria in previous cultures and depict a higher risk for bronchiectasis if sensitized to rAsp f1 or rAsp f3. Despite considerably lower vitamin D levels in COPD compared to controls, we did not find any relation with sensitization to *A. fumigatus* or bronchiectasis.

In our cohort, 34.3% of COPD patients had increased levels of total IgE compared to 22% in the control group. The higher prevalence of increased total IgE (47.3%) reported in another COPD cohort (7) may be explained by demographical differences, variable inclusion criteria and the use of a lower cut-off for positivity. In terms of sensitization to *A. fumigatus*, we found an 18.3% prevalence in COPD when taking crude extract and recombinant antigens into account. These results are in line with previous reported data of prevalence between 8.5 and 16% (6,7,33,34). As these studies are lacking an appropriate control group, our data clearly establish that sensitization to *A. fumigatus* is more common in COPD. We are aware of the younger age in our control group, but a median age difference of only 5 years seems insufficient to explain the higher rate of sensitization in COPD. The lack of skin prick tests is a limitation of our study because of a potential discordance between in vitro tests and skin prick tests to diagnose *A. fumigatus* sensitization (35).

Bafadhel et al showed an association between sensitization and lower FEV1 in COPD (6). Although our sample size was twice as large, we could not observe any relationship with FEV1, diffusion capacity, exacerbations or GOLD stages. A more pronounced male predominance in the sensitized group may seem surprising, but has been described before (36,37). Cigarette smoke exposure is known to facilitate development of sensitization, however we found no difference in pack-years and current smoking between *A. fumigatus* sensitized and non-sensitized COPD patients. This is in contrast to a recent paper about bidi smokers where current smoking seemed a risk factor for *A. fumigatus* sensitization, irrespective of the presence of COPD (34).

Despite similar disease severity, bronchiectasis was significantly more frequent in sensitized patients, particularly when sensitized to rAsp f1 or rAsp f3 (50% and 60% prevalence,

respectively). Moreover, multivariate analysis showed that the associated risk for bronchiectasis was independent of gender, age, BMI, diffusion capacity, frequent exacerbations and presence of PPM. Together, these observations support the hypothesis that *A. fumigatus* hypersensitivity might contribute to the development of bronchiectasis in COPD. Based on our results we are not able to attribute causality. Furthermore, we realize that the association exists in two directions since the presence of bronchiectasis and COPD is a risk factor for colonization with *A. fumigatus* due to reduced mucociliary clearance, therapy with (inhaled) corticosteroids, antibiotics etc. Nevertheless, some previous papers also suggested *Aspergillus fumigatus* as a causal factor for bronchiectasis. Clinically, colonization by *A. fumigatus* was associated with higher sputum volumes in bronchiectasis (38) and development of bronchiolitis obliterans syndrome after lung transplantation (39). Furthermore, *A. fumigatus* sensitization was associated with bronchiectasis irrespective of ABPA in asthma (40) and total IgE, which may represent an indirect marker of sensitization, seemed a risk factor for bronchiectasis in COPD patients (41). From a mechanistic perspective, Aspergillus proteases play an important role. The serine protease activity of *A. fumigatus* stimulates MUC5AC, resulting in more mucus production by epithelial cells (42) and *A. fumigatus* allergen proteases Asp f5 and Asp f13 have been shown responsible for stimulation of the Th2 pathway and airway remodelling in a murine model (43).

We could not measure blood eosinophils on the frozen blood samples, and therefore we could not fulfill criteria for ABPA in this cohort (44). In a recent paper, *A. fumigatus* IgG >27mg/L was used in a cohort of sensitized asthma patients to distinguish patients having ABPA from patients without ABPA (45). This cut-off seems inappropriate in our *A. fumigatus* sensitized COPD patients because the median *A. fumigatus* IgG value in our sensitized group was 43.2 mg/L. This could be explained by the fact that specific IgG is dependent on region, exposure and underlying disease. The higher levels of *A. fumigatus* specific IgG, as a measure of exposure, may be due to reduced mucociliary function and reduced *A. fumigatus* clearance by innate immune cells in COPD versus controls (46). This reduced clearance of fungal allergens may facilitate sensitization.

Next to the higher prevalence of bronchiectasis, more *A. fumigatus* sensitized COPD patients had *Pseudomonas aeruginosa* and *Serratia marcescens* present in their sputum compared to *A. fumigatus* non-sensitized COPD patients. This finding further emphasizes the clinical

relevance of our results since these pathogens may play a role in exacerbations and can become multidrug resistant. We did not report on culture results of *A. fumigatus* because the specific growth medium (Sabouraud) was only used on clinical indication. Moreover, sensitivity of culture for *A. fumigatus* is low and other detection methods such as PCR may better reflect the presence of *A. fumigatus* in the airways (47). Further prospective studies are therefore needed to explore the relationship between *A. fumigatus* colonization, sensitization and bronchiectasis. In line with these unknowns is the potential role of *A. fumigatus* on vitamin D receptor downregulation (20) as well as the effect of vitamin D deficiency on *A. fumigatus* sensitization (19,21) and disease severity in bronchiectasis (22). Our results did not show any obvious relationship, but it is clear that we cannot rule out a role of vitamin D deficiency based on this cross-sectional analysis with relative small sample size.

We are the first to report data on IgE against recombinant *A. fumigatus* allergens in COPD. rAsp f1 is a major and species-specific allergen of *A. fumigatus*, which in contrast to *A. fumigatus* extract shows no cross-reactivity with proteins of other species (8). Exposure to rAsp f1 only occurs during fungal growth which makes rAsp f1 a reliable and relevant antigen (48). Although testing with the crude extract is assumed more sensitive than testing with the recombinants, we showed a higher prevalence of sensitization to rAsp f1 compared to the crude extract. This finding questions the reliability of the crude extract to diagnose *A. fumigatus* sensitization and emphasizes its known limitations such as cross-reactivity, lability and lack of standardization. However, it remains unclear why sensitization to rAsp f2 and rAsp f3 (other major allergens of *A. fumigatus*) was less frequently detected. Several reasons could explain why bronchiectasis were not present in the patients only sensitized against the crude *A. fumigatus* extract. The 9 patients only sensitized to the crude *A. fumigatus* extract may be false positive due to cross-reactivity. On the other hand, specific allergens may be required to trigger pathways that contribute to bronchiectasis development, although controversial in ABPA (10,49). The role of different *A. fumigatus* allergens in the pathogenesis of ABPA and bronchiectasis is far from understood and more than 25 distinct *A. fumigatus* allergens have been identified.

The prevalence of bronchiectasis in our cohort (33%) lies within the wide range reported in other studies (4 to 60%) (15,50,51). Although there is no specific radiologic scoring system

validated for bronchiectasis in COPD, the modified Reiff score which we applied, has been used in several studies (29,52,53). Nevertheless, overestimation of bronchiectasis in COPD is possible since limited bronchiectasis are seen on high resolution CT scans of healthy individuals and are also related to lung aging (30,54). We tried to avoid overestimation by considering only a modified Reiff ≥2 as meaningful and showed that the prevalence of bronchiectasis was at least higher in our patient cohort than in the controls.

These results show a high prevalence of *A. fumigatus* sensitization in COPD patients and highlight a potential role of rAsp f1 and rAsp f3 in COPD-related bronchiectasis. Because of the retrospective, observational design of this study, we were not able to demonstrate a causal relationship. Nevertheless, this is the first report about the association between *A. fumigatus* sensitization, including 5 recombinant antigens, and bronchiectasis in COPD. An association between *A. fumigatus* sensitization and vitamin D deficiency was not found. The search for underlying mechanisms in the development of bronchiectasis in COPD is relevant, since the presence of bronchiectasis in COPD influences management and prognosis of the disease. Prospective, longitudinal studies are needed to proof causality and to allow speculation about preventive interventions.

Disclosure

The authors declare that they have no relevant competing interests.

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B. *Aspergillus fumigatus* detection and risk factors in patients with COPD-bronchiectasis overlap

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ABSTRACT

Background

The role of *Aspergillus fumigatus* in the airways of chronic obstructive pulmonary disease (COPD) patients with bronchiectasis is currently unclear. We searched for a sensitive and noninvasive method for *A. fumigatus* detection in the sputum of COPD patients and addressed potential risk factors for its presence.

Methods

Induced sputum samples of 18 COPD patients and 17 COPD patients with bronchiectasis were analyzed for the presence of *A. fumigatus* by culture, galactomannan detection, and PCR.

Results

Of the patients with COPD–bronchiectasis overlap, 23.5% had a positive culture for *A. fumigatus* versus 10.5% of COPD patients without bronchiectasis ($p = 0.39$). The median sputum galactomannan optical density index was significantly higher in patients with COPD and bronchiectasis compared with patients with COPD alone ($p = 0.026$) and ranged between the levels of healthy controls and *A. fumigatus*-colonized cystic fibrosis patients. Both the presence of bronchiectasis and the administration of systemic corticosteroids were associated with sputum galactomannan ($p = 0.0028$ and $p = 0.0044$, respectively) and showed significant interaction (p interaction = 0.022). PCR for *Aspergillus* was found to be a less sensitive method, but was critically dependent on the extraction technique.

Conclusion

The higher sputum galactomannan levels suggest a more abundant presence of *A. fumigatus* in the airways of patients with COPD–bronchiectasis overlap compared with patients with COPD without bronchiectasis, particularly when systemic corticosteroids are administered.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a highly prevalent disease, characterized by progressive airflow limitation, which is caused by an abnormal inflammatory response to chronic inhalation of irritants, mainly cigarette smoke (1). Both small airways (obstructive bronchiolitis) and parenchyma (emphysema) are affected. Bronchiectasis is defined by the presence of irreversibly dilated and chronically inflamed bronchi. COPD and bronchiectasis have overlapping clinical features, but the diagnostic criteria are very different. Whereas an irreversible obstructive lung function is obligatory in COPD, bronchiectasis is diagnosed by structural airway abnormalities observed on computed tomography (CT) of the thorax. The combined presence of COPD and bronchiectasis in one patient is not rare. In cohorts of COPD patients, bronchiectasis has been described in 30–60% of patients (2–5), whereas bronchiectasis cohorts report COPD as the underlying cause in approximately 30% of patients (6,7). The recognition of an overlap is relevant, since these patients not only have a worse prognosis (2,7,8), but their diagnostic and therapeutic approach is also different.

The pathogenesis of bronchiectasis is generally explained by a vicious circle of inflammation, structural damage, impaired mucus clearance, bacterial colonization, and infection (9). The underlying mechanism for the development of bronchiectasis in a subset of COPD patients remains largely unknown. *Aspergillus fumigatus* is a ubiquitous fungus, which is cleared from the airways by the innate immune system. A damaged airway epithelium, inherited or acquired defects of the innate immune system, and immunosuppressive drugs such as corticosteroids (CS) predispose to aspergillosis. *A. fumigatus* colonization and sensitization associate with bronchiectasis and unfavorable outcomes in asthma and cystic fibrosis (CF) (10–13). In COPD, a history of exacerbations and the isolation of pathogenic bacteria in sputum were suggested as potential risk factors for *A. fumigatus* (14). Although the clinical relevance is not clear, several retrospective studies showed that more than 20% of COPD patients with *A. fumigatus* detected in respiratory samples eventually developed aspergillosis (15,16). If *A. fumigatus* plays a causal role in the development of bronchiectasis is still a matter of debate.

One major reason for our limited understanding is the lack of sensitive and noninvasive methods to detect *A. fumigatus* in the airways. Mycological culture is not sensitive nor

standardized and has led to an underestimation of *A. fumigatus* in the airways (17). Other methods focus mainly on invasive fungal disease and require invasive procedures, such as immunohistochemistry on airway biopsies or galactomannan detection on bronchoalveolar lavage fluid. Galactomannan is a polysaccharide cell wall component of *Aspergillus species*, which is not present in resting conidia but is secreted by the hyphae during fungal growth. The determination of galactomannan by enzyme immunoassay in serum and bronchoalveolar lavage fluid is validated for diagnosing an invasive *Aspergillus* disease, mainly in immunocompromised patients. Molecular-based techniques such as polymerase chain reaction (PCR) and next-generation sequencing are hampered by complex DNA extraction, high costs, and limited experience with sputum samples. To investigate the role of *A. fumigatus* in patients with COPD, we explored the feasibility of galactomannan detection and *A. fumigatus* PCR on sputum samples.

We assumed a more abundant presence of *A. fumigatus* in the sputum of patients with COPD–bronchiectasis overlap and addressed the role of CS as a potential risk factor.

MATERIALS AND METHODS

Study design and subjects

This academic, single-center study included COPD patients prospectively during hospitalization or an outpatient visit. The inclusion criteria were an established diagnosis of COPD based on a post-bronchodilator forced expiratory volume in 1 s (FEV1)/forced vital capacity (FVC) ratio < 0.7, smoking history of at least 10 pack-years, available CT images of the thorax, and an FEV1 ≥ 30% (safety measure for sputum induction). The exclusion criteria were ventilation (mechanical and noninvasive), respiratory diagnosis other than COPD, mycobacterial disease, immunosuppression other than CS, active cancer treatment, history of lung, tracheal, or laryngeal surgery, history of chest radiotherapy, and presence of other inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease. Investigators were blinded to the results of previous sputum cultures. None of the included patients had a history of allergic or chronic bronchopulmonary aspergillosis, nor invasive *Aspergillus* disease. The presence of bronchiectasis was assessed on CT scans of the lungs. All hospitalized patients received standard therapy for an exacerbation: CS and antibiotics if indicated. They were included after sufficient recovery from the acute event. Oral or intravenous CS and antibiotics administered in the seven days before sputum induction were considered relevant. The study was approved by the local Ethics Committee (Medical Ethical Board of the University Hospitals Leuven, Belgium—M11223) and all patients signed informed consent before enrollment.

Pulmonary function and questionnaires

Post-bronchodilator spirometry was measured using standardized equipment (Whole Body Plethysmograph, Vyaire, Vilvoorde, Belgium), according to the American Thoracic Society/European Respiratory Society guidelines (26). Diffusion capacity was measured by the single-breath carbon monoxide gas transfer method (27). The results are reported as percentages predicted of reference values. The post-bronchodilator FEV1 was used to classify the patients according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification. The patients completed the modified Medical Research Council (mMRC)

breathlessness scale (28), COPD Assessment Test (CAT™) (29), and Saint Georges Respiratory Questionnaire (SGRQ), a self-administered health-related quality of life measure.

CT Thorax

All subjects had a high-resolution CT (HRCT) of the thorax at least one year before enrollment. All patients had inspiratory images with 1 mm slices. Bronchiectasis were defined based on Naidich's descriptions: bronchoarterial ratio > 1, lack of tapering, and presence of bronchus within 1 cm of the costal pleura or abutting the mediastinal pleura (30). All images were blinded to the other data and scored using the modified Reiff score, assessing the number of involved lobes (the lingula considered separately) and the degree of bronchodilation (1 = tubular, 2 = varicose, and 3 = cystic) (31).

Eosinophils, Total IgE, A. fumigatus Sensitization, and A. fumigatus-Specific IgG

Blood samples were collected, and white blood cell count and differentiation were performed by fluorescence flow cytometry (Sysmex XE-5000, Kobe, Japan). Total IgE, IgE against *A. fumigatus* extract, recombinant antigens (rAsp f1-f4 and f6), and *A. fumigatus* IgG were determined by ImmunoCAP fluoroenzyme-immunoassay (Phadia AB, Uppsala, Sweden). The skin prick test with crude *A. fumigatus* extract (ALK, Almere, The Netherlands) was performed according to the guidelines (32).

Sputum Collection, homogenization, and culture

In all COPD patients, sputum was induced by inhalation of hypertonic saline (concentration 3%, 4%, and 5% for 5 min each) generated by an Ultra-Neb ultrasonic nebulizer (DeVilbiss, Port Washington, NY, USA) after pretreatment with 400µg of inhaled salbutamol. The patient was asked to rinse the mouth thoroughly with water and spit the sputum into a collection tube (33). As negative and positive control samples, induced sputum samples of 7 healthy controls and spontaneous samples of 12 CF patients with known *A. fumigatus* colonization were collected, respectively. All samples were immediately stored at −80 °C. For homogenization, an equal volume of Sputasol® (Oxoid, Thermo Fisher, Hampshire, UK, 1.4% dithiothreitol) was added to each sample. The samples were subsequently incubated at 37 °C for 30 min and shaken every 10 min. Two volumes of each

sample, 10 and 100µL, were inoculated on Sabouraud agar plates (Sabouraud with chloramphenicol (Bio-rad, Marnes-la-Coquette, France). The culture plates were incubated at 42 °C for 1 day and subsequently at 30 °C for 1 week. For each sample, an additional plate without inoculation was incubated as a control. The growth was checked daily and the colonies were identified by microscopy.

Sputum galactomannan assay

Of the homogenized sputum, 300µL was transferred to a sterile tube. An enzyme immunoassay (Platelia™ *Aspergillus* Ag, Bio-Rad, Marnes-la-Coquette, France) was used to detect galactomannan. The results are expressed as optical density index (ODI), the ratio of the optical density of the sample to the mean cut-off control optical density. To exclude false positivity, galactomannan was determined in Sputasol® and in the sterile water we used to prepare the Sputasol® solution. Furthermore, three vials from different batches of amoxicillin clavulanate were tested, as some of the hospitalized patients received this antibiotic, known to influence galactomannan results in previous reports (34). Moreover, 13 sputum samples were retested to confirm the results.

Aspergillus PCR on Sputum

A. fumigatus real-time PCR was done following an in-house method with excellent performance in European *Aspergillus* PCR Initiative (EAPCRI) evaluations. Nucleic acid extraction was semi-automatically performed with EasyMAG (Biomérieux, Marcy l' Etoile, France) using NucliSens reagents as recommended by the manufacturer. Briefly, 500µL of sputum samples was lysed in the presence of magnetic silica beads and subsequently eluted. This method was compared with a manual extraction method using MycXtra® (Myconostica, Cambridge, UK) in five additionally collected CF sputum samples with known *A. fumigatus* colonization. The fungal DNA was extracted as recommended by the manufacturer. Briefly, 1 mL of the homogenized sample was used for extraction, then lysis, bead beating, and purification were performed to remove inhibiting substances with reagents provided by the manufacturer. The nucleic acid extracts where used for real-time PCR (Quantstudio™, Thermo Fisher Life technologies, Carlsbad, CA, USA). The comparative cycle threshold was

used to normalize data after running through 45 cycles. The used reagents are shown in the online supplement, Table S3.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA) and SAS software version 9.4 (SAS Institute, Cary, NC, USA). Non-parametric tests were used for analyses. Univariate comparisons were performed by Mann–Withney U-test and presented as median ± interquartile range. The proportions of discrete variables were compared with χ^2 test and presented as absolute numbers and percentages. The comparison of four groups was performed with the Kruskal–Wallis and post-hoc Dunn's tests for multiple comparison. A multivariate model was built for two-way analysis of variance to study the association between sputum galactomannan, bronchiectasis, and CS. After performing bivariable models with sputum galactomannan as exposure, potential confounders of the association between sputum galactomannan and bronchiectasis were included in the final model if they (1) changed the estimate of the multivariable model ≥10% or (2) the variable was significantly associated with galactomannan and bronchiectasis. The interaction between bronchiectasis and CS was included in the model. *p*-values < 0.05 were considered significant in all analyses.

RESULTS

Study group characteristics

Thirty-six COPD patients were included, of which 17 had bronchiectasis, with a median modified Reiff score of 4. There were no significant differences between the two groups, apart from a significantly lower median value of pack-years (p = 0.048) in patients with bronchiectasis. The results are shown in Table 1.

Table 1: Characteristics of the study groups.

Characteristic	COPD without bronchiectasis	COPD with bronchiectasis
Subjects, *n*	19	17
Hospitalized, %	42	65
Age, y	71 (60–75)	68 (63–79)
Male, %	84	88
BMI, kg/m²	25 (20–30)	22 (19–27)
Pack-years *	48 (40–55)	40 (25–45)
FEV1, % pred	51 (39–58)	41 (37–52)
DLCO, % pred	47 (40–57)	44 (26–53)
GOLD		
I/II/III/IV, %	0/53/47/0	6/29/65/0
A/B/C/D, %	21/0/16/63	12/17/6/65
Eosinophils, %	1.4 (0.1–3.1)	2.3 (0.8–4.6)
Eosinophils, µL	200 (0–300)	200 (100–350)
Total IgE, kU/L	83 (16–592)	43 (33–298)
mMRC	2 (1–2)	2 (1.5–3.5)
CAT	14 (12–20)	19 (13–24)
SGRQ	41.9 (30.6–53.4)	54.9 (37.3–66.7)
≥2 exacerbations/y, %	79	71
ICS, %	89	82
Modified Reiff score	NA	4 (2.5–6.5)

Data are presented as *n*, % or median (interquartile range). COPD: chronic obstructive pulmonary disease, y: year, BMI: body mass index, FEV1: forced expiratory volume in 1 s, % pred: percentage predicted, DLCO: diffusion capacity of the lung for carbon monoxide, GOLD: Global initiative for chronic Obstructive Lung Disease stadia, mMRC: modified Medical Research Council breathlessness scale, CAT: COPD Assessment Test, SGRQ: Saint Georges Respiratory Questionnaire, ICS: inhaled corticosteroids. * p-value < 0.05. All other variables were not significantly different between the groups.

A. fumigatus antibodies

Patients with COPD–bronchiectasis overlap had a median *A. fumigatus* IgG value of 35.4 mg/L versus 17.4 mg/L in COPD patients without bronchiectasis (p = 0.14). On the basis of both ImmunoCAP measurement and skin prick test, three patients without (15.7%) and five patients with bronchiectasis (29.4%) were sensitized to *A. fumigatus* (p = 0.37). The results are summarized in Table 2.

Table 2: *A. fumigatus*-related results.

Test	COPD without bronchiectasis	COPD with bronchiectasis	*p*-Value
Subjects, *n*	19	17	
A. fumigatus IgG, mg/L	17.4 (13.4–37.3)	35.4 (25.5–51.6)	0.14
A. fumigatus sensitization, %	15.7	29.4	0.37
A. fumigatus sputum culture, %	10.5	23.5	0.39
Sputum galactomannan, ODI	0.7 (0.4–1.5)	3.7 (0.6–5.7)	**0.026**

Data are presented as *n*, median (interquartile range) or %. COPD: chronic obstructive pulmonary disease, *A. fumigatus: Aspergillus fumigatus*, ODI: optical density index. *p*-value < 0.05 is captured in bold.

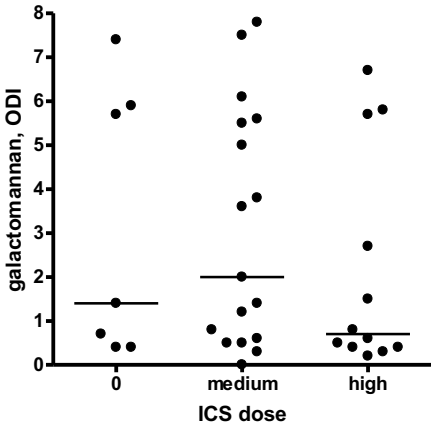
Fungal sputum culture

Four COPD patients with bronchiectasis (23.5%) had a positive *A. fumigatus* culture versus two COPD patients without bronchiectasis (10.5%) ($p = 0.39$). *Aspergillus nidulans* was found in samples of two COPD–bronchiectasis-overlap patients which were also positive for *A. fumigatus*. When only inocula of 10 µL were considered, three out of six positive cultures were negative. Of the non-inoculated control plates and of the healthy control samples, none grew *A. fumigatus*, but a positive *A. fumigatus* culture was confirmed in all samples of cystic fibrosis patients with previously known *A. fumigatus* colonization.

Sputum galactomannan

The median sputum galactomannan ODI was significantly higher in patients with COPD–bronchiectasis overlap compared with patients with only COPD (3.7 versus 0.7, $p = 0.026$) (Table 2). The results of galactomannan measurements in Sputasol, sterile water, and three vials of amoxicillin clavulanate were 0.0, 0.1, and 0.0 respectively. Thirteen sputum samples were retested and had a median galactomannan inter-assay difference of 0.4, with a median coefficient of variation of 9.4% (Supplement Table S1). The majority of patients was using inhalation corticosteroids (ICS) (Table 1); we found no difference in sputum galactomannan between patients without ICS or with medium or high dose ICS (Figure 1).

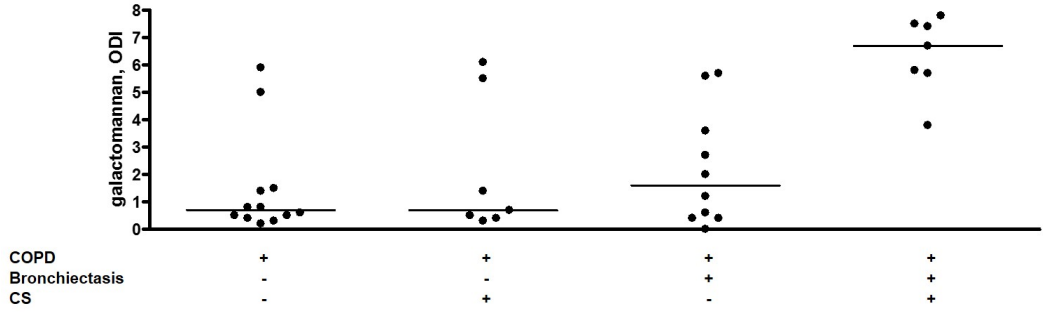
Figure 1: Inhaled corticosteroids and sputum galactomannan.



Sputum galactomannan is presented as optical density index. Every dot represents one sample, and the horizontal lines reflect the median values. There was no significant difference in galactomannan values dependent on ICS dose. ODI: optical density index, ICS: inhaled corticosteroids.

In contrast to ICS, both bronchiectasis and systemic CS were significantly associated with sputum galactomannan ODI (p = 0.0028 and p = 0.0044 respectively) in a two-way analysis of variance model. Furthermore, CS showed a stronger association with galactomannan in the patients with COPD–bronchiectasis overlap compared with COPD patients without bronchiectasis (p interaction = 0.022). The results are presented in Figure 2 and Table 3.

Figure 2: Effect of systemic corticosteroids on sputum galactomannan values.



Sputum galactomannan is presented as optical density index. Every dot represents one sample, and the horizontal lines reflect the median values. ODI: optical density index, COPD: chronic obstructive pulmonary disease, CS: systemic corticosteroids in the previous seven days.

Table 3: Associations with sputum galactomannan in COPD patients.

Bivariate Models		
	Estimate	***p*-Value**
CS in COPD without bronchiectasis (*n* = 19)	0.64	0.54
CS in COPD with bronchiectasis (*n* = 17)	4.17	**0.0004**
Multivariate Model		
	Estimate	***p*-Value**
Bronchiectasis	0.67	**0.0028**
CS	0.57	**0.0044**
Antibiotics	0.37	0.63
Bronchiectasis–CS interaction	3.43	**0.022**

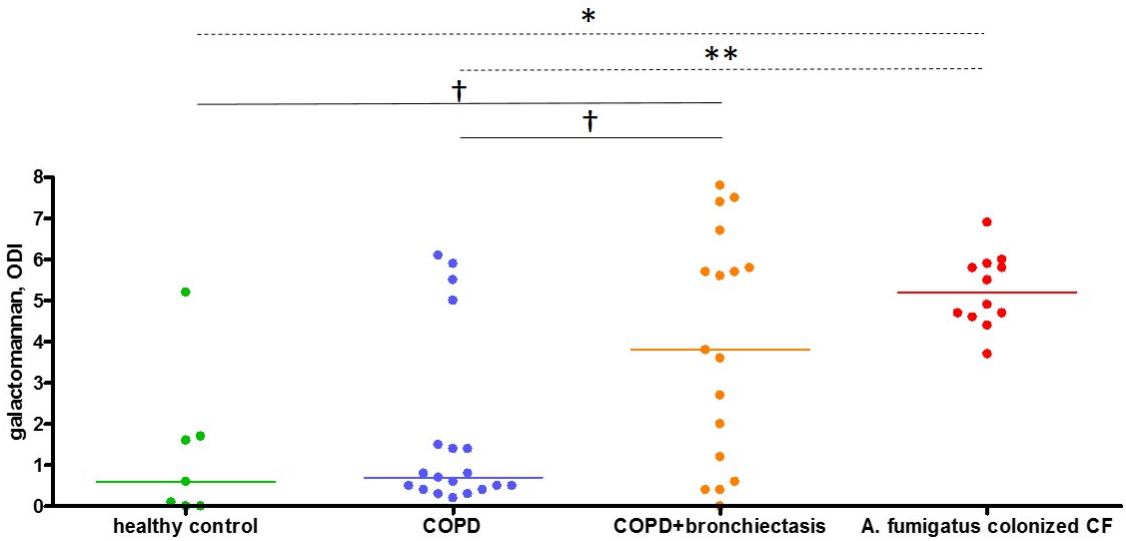
Two bivariate general linear models were built with galactomannan as exposure: one for patients without and one for patients with bronchiectasis showing that CS were only significant associated with galactomannan in patients having bronchiectasis. One multivariate (two-way analysis of variance) general linear model was built for all patients in which systemic corticosteroids and antibiotics administered in the seven days before sputum induction were considered relevant. CS: systemic corticosteroids, COPD: chronic obstructive pulmonary disease, *p*-values < 0.05 are shown in bold.

As galactomannan detection on sputum has not been validated, we compared our results to negative and positive control samples and found them to be significantly different (p = 0.0012, Figure 3). Moreover, all but one subjects with a positive *A. fumigatus* culture had a sputum galactomannan ODI above 4 (Supplement Table S2).

Sputum A. fumigatus PCR

When automated DNA extraction was used for *A. fumigatus* real-time PCR, only three samples of COPD patients were found to be positive. All of them had high sputum galactomannan levels. All healthy control samples had a negative PCR result, whereas seven out of twelve CF samples with known *A. fumigatus* colonization had a positive PCR result. Five positive control sputum samples were used to compare automated DNA extraction by EasyMAG and manual extraction by MycXtra®. All samples had a positive *A. fumigatus* PCR result after manual DNA extraction compared to only two positive samples after the automated extraction protocol (Table 4). The comparison between sputum galactomannan and *A. fumigatus* PCR is shown in Table S2 of the supplement.

Figure 3: Comparison of COPD sputum galactomannan with healthy and *A. fumigatus*-colonized CF controls.



Sputum galactomannan is presented as optical density index. Every dot represents one sample, and the horizontal lines reflect the median values. Kruskal–Wallis test showed an overall significant difference (p = 0.0012), with Dunn's post-hoc comparison for multiple testing showing significant differences between groups, indicated with * $p < 0.05$ and ** $p < 0.01$ (dashed lines). The comparison of COPD + bronchiectasis with the other groups using Mann–Withney U-test showed significant differences between groups, indicated with † $p < 0.05$ (solid lines). ODI: optical density index, COPD: chronic obstructive pulmonary disease, CF: cystic fibrosis.

Table 4: Comparison of DNA extraction methods in five samples of cystic fibrosis patients with known *A. fumigatus* colonization.

Subject	Sputum Galactomannan, ODI	PCR after EasyMAG Extraction, Ct	PCR after MycXtra® Extraction, Ct
CF1	4.4	negative	positive, 36.7
CF2	3.7	negative	positive, 32.9
CF3	4.7	positive, 29.3	positive, 26.2
CF4	4.6	positive, 30.7	positive, 30.5
CF5	4.7	negative	positive, 33.2

Sputum galactomannan is presented as optical density index. PCR is expressed as negative or positive with the respective Ct value in case of positivity. CF: cystic fibrosis sample, ODI: optical density index, PCR: polymerase chain reaction, Ct: cycle threshold.

DISCUSSION

Sputum galactomannan levels suggest a higher load of *A. fumigatus* in COPD-bronchiectasis-overlap patients compared to COPD patients without bronchiectasis. The sputum galactomannan values were particularly higher in COPD-bronchiectasis-overlap patients that received systemic CS.

Fungal culture has a low sensitivity, even in proven aspergillosis, which contributes to an underestimation of fungal presence in the airways (18). In this study, 16.7% of all COPD patients showed *A. fumigatus* in sputum culture. A positive culture was not associated with clinical characteristics or bronchiectasis. Previous studies reported *A. fumigatus* positive cultures in 14–37% of COPD patients (14,19). In general, culture procedures are not standardized, and the use of different media and methodologies makes it hard to compare different studies. On the basis of a recent report that emphasized the use of larger inocula for fungal culture, we compared sputum volumes of 10 µL and 100 µL (20). Indeed, we found a higher *A. fumigatus* culture yield by plating 100 µL of the samples. Nevertheless, the lack of standardization and low sensitivity urges the need for other detection methods.

An attractive, alternative method is the detection of galactomannan on sputum samples. On bronchoalveolar lavage fluid, a galactomannan value of 0.5 is suggestive of the presence of *A. fumigatus*, although this cut-off is under debate. Kimura et al. reported 1.2 as the optimal sputum galactomannan cut-off to diagnose invasive aspergillosis in haematological patients (21), whereas Baxter et al. used the 0.5 cut-off for *Aspergillus* positivity in adult CF sputum samples (22). Recently, the sputum galactomannan results of patients with chronic pulmonary aspergillosis and allergic bronchopulmonary aspergillosis were found to be much higher, with a cut-off above 6.5 for diagnosis (23). Our study, which used a comparable homogenization of the samples as the latter, found ODI values in the same range. Furthermore, we validated these results with negative and positive controls, which were confirmed by culture and PCR. Despite the higher median galactomannan ODI in COPD patients with bronchiectasis, our study was not able to define an appropriate cut-off. The median coefficient of variation to evaluate inter-assay difference was acceptable, although we are aware that the inter-assay difference was not optimal for some of our samples.

A. fumigatus PCR after automated DNA extraction was less sensitive than culture and galactomannan detection on COPD sputum samples. Although the performance is good for clear, liquid samples, our in-house protocol was not suitable for sputum samples, as it does not apply mechanical forces prior to fungal DNA extraction. Manual DNA extraction with MycXtra, which includes bead-beating, performed much better in five additionally collected positive control samples. Since this method required a high sample volume, we were not able to apply this on the other, previously collected study samples. Furthermore, manual extraction is labor-intensive and not suited for large laboratories handling many samples on a daily basis. Any protocol for fungal DNA extraction and PCR on sputum needs further optimization prior to its clinical use.

CS temper the innate immune response, thereby permitting *A. fumigatus* to persist and proliferate. In our study, systemic administration of CS was significantly associated with higher sputum galactomannan values, particularly in patients with COPD–bronchiectasis overlap. These findings contrast with the study of Huerta et al., in which no association was found between *Aspergillus* cultures and oral CS (14). Differences in the studied population or in the dose of CS administered prior to the analysis may explain these discrepancies. As the large majority of our study subjects on CS received high doses in the context of acute exacerbation (40 mg prednisolone), dose–response relationships could not be studied. Together, our data suggest that systemic CS induce a pronounced and rapid growth of residing *A. fumigatus* in the airways, which may predispose to sensitization, bronchiectasis development, or fungal infection. A local deposition of CS via inhalation may have similar effects. Bafadhel et al. found that COPD patients with *A. fumigatus*-positive culture were on higher doses of inhaled CS compared with culture-negative patients (19). However, the significance of this association was weak and could not be confirmed by others (14) nor by our analysis.

Although this study does not allow us to attribute causality, our findings support the hypothesis that *A. fumigatus* contributes to the development and/or progression of bronchiectasis in COPD. We recently showed that the sensitization to specific *A. fumigatus* allergens was associated with the presence of bronchiectasis in COPD (5). Next to an upregulation of the Th2 pathway, *Aspergillus* proteases may play an important role through enhanced mucus production and airway remodeling (24,25). So far, studies that explored these relationships are solely based on fungal cultures, which may explain some of the

inconclusive results. Sputum galactomannan seems to provide a more sensitive marker for *A. fumigatus* detection in the airways of COPD patients. This advantage may serve new longitudinal observational studies or even intervention trials in this area.

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Author Contributions

Wim Janssens, Katrien Lagrou, Lieven J. Dupont, Bart M. Vanaudenaerde, and Stephanie Everaerts conceived and designed the experiments, Stephanie Everaerts performed the experiments, Stephanie Everaerts analyzed the data, Stephanie Everaerts, Kristina Vermeersch, and Wim Janssens wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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SUPPLEMENTAL MATERIAL

Table S1: inter-assay difference in 13 sputum samples

Galactomannan 1, ODI	Galactomannan 2, ODI	Difference	CV, %
0.5	0.9	0.4	40.4
0.2	0.2	0	0
5.5	6.3	0.8	9.6
0.3	0.3	0	0
0.7	0.8	0.1	9.4
1.4	1.4	0	0
7.4	6.3	-1.1	11.4
6.7	6.1	-0.6	6.6
3.5	5.2	1.7	27.6
7.8	6.2	-1.6	16.2
2.7	2.5	-0.2	5.4
7.5	6.2	-1.3	13.4
5.6	5.8	0.2	2.5

Galactomannan measurement on same samples of 13 subjects. Galactomannan is presented as optical density index ODI: optical density index, CV: coefficient of variation.

Table S2: *Aspergillus* sputum culture, sputum galactomannan and PCR per patient

	A. Fumigatus culture	Sputum galactomannan, ODI	*A. Fumigatus* PCR, Ct
COPD 1	-	0.5	-
COPD 2	-	0.2	-
COPD 3	positive	5.5	29.6
COPD 4	-	0.3	-
COPD 5	-	0.7	-
COPD 6	-	1.4	-
COPD 7	-	0.4	-
COPD 8	-	0.5	-
COPD 9	-	1.5	-
COPD 10	-	0.8	-
COPD 11	-	5	-
COPD 12	-	0.3	-
COPD 13	-	5.9	-
COPD 14	-	0.6	-
COPD 15	positive	6.1	27.1
COPD 16	-	0.5	-
COPD 17	-	0.8	-
COPD 18	-	0.4	-
COPD 19	-	1.4	-
COPD+bronchiectasis 1	-	7.4	-
COPD+bronchiectasis 2	positive	6.7	31.1
COPD+bronchiectasis 3	-	3.6	-
COPD+bronchiectasis 4	positive	7.8	-
COPD+bronchiectasis 5	-	2.7	INH
COPD+bronchiectasis 6	positive	7.5	-

COPD+bronchiectasis 7	-	5.6	-*
COPD+bronchiectasis 8	-	0.4	-
COPD+bronchiectasis 9	-	3.8	-
COPD+bronchiectasis 10	-	5.8	-
COPD+bronchiectasis 11	-	5.7	-*
COPD+bronchiectasis 12	-	0.4	-
COPD+bronchiectasis 13	-	0	-
COPD+bronchiectasis 14	-	0.6	-
COPD+bronchiectasis 15	-	5.7	-
COPD+bronchiectasis 16	positive	2	-
COPD+bronchiectasis 17	-	1.2	-
negative control 1	-	1.6	-
negative control 2	-	0	-
negative control 3	-	1.7	-
negative control 4	-	0	-
negative control 5	-	5.2	-
negative control 6	-	0.6	-
negative control 7	-	0.1	-
positive control 1	positive	6.9	35.2
positive control 2	positive	5.8	27.6
positive control 3	positive	4.9	33.3
positive control 4	positive	5.5	-
positive control 5	positive	5.8	29.4
positive control 6	positive	6	-
positive control 7	positive	5.9	30.8
positive control 8	positive	4.4	-
positive control 9	positive	3.7	-
positive control 10	positive	4.7	29.3
positive control 11	positive	4.6	30.7
positive control 12	positive	4.7	-

Galactomannan is presented as optical density index. *A. fumigatus* PCR was performed after automated DNA extraction. If PCR was positive, a Ct value is presented. COPD: chronic obstructive pulmonary disease, ODI: optical density index, PCR: polymerase chain reaction, Ct: cycle threshold, INH: inhibition despite of dilution in second run, *: diluted 1/5 because of inhibition in first run.

Table S3: used reagents for real-time PCR

Target	28S rRNA
Primer, forward	5'-GCACGTGAAATTGTTGAAAGG-3'
Primer, reverse	5'-CAGGCTGGCCGCATTG-3'
Probe	FAM-5'-CATTCGTGCCGGTGTACTTCCCCG-3'-TAMRA
Polymerase	TaqMan Fast Virus Mix (Life technologies, Carlsbad, USA)
Temperature program	95°C – 20 seconds

Chapter 4

Airway morphometry in COPD with bronchiectasis: a view on all airway generations

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ABSTRACT

Background

The pathophysiological processes underlying bronchiectasis in COPD are not understood. In COPD, both small and large airways are progressively lost. To what extend the different airway generations of patients with COPD and bronchiectasis are involved, is currently not known.

Materials and methods

7 COPD explant lungs with bronchiectasis were compared to 7 COPD explant lungs without bronchiectasis and 7 unused donor lungs as controls. In order to investigate all airway generations, a threefold imaging approach with different resolutions was conducted. Per group, 5 lungs were frozen (n=15), underwent CT imaging for large airway evaluation and 4 tissue cores per lung were taken for detailed microCT analysis of the terminal bronchioles. Two other lungs per group (n=6) were air-dried to obtain lobar microCT images allowing airway segmentation and 3D quantification of the airway tree.

Results

COPD lungs with bronchiectasis had significantly more airways compared to COPD lungs without bronchiectasis ($p<0.0001$), with large airway numbers similar to control lungs. This difference was present in both upper and lower lobes. Lack of tapering was present ($p=0.010$) and larger diameters were demonstrated in lower lobes with bronchiectasis ($p=0.0098$). MicroCT analysis of tissue cores showed similar reductions of tissue percentage, surface density and number of terminal bronchioles in both COPD groups compared to control lungs.

Conclusion

Although terminal bronchioles were equally reduced in COPD lungs with and without bronchiectasis, significantly more large and small airways were found in COPD lungs with bronchiectasis.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a prevalent disease, characterized by persistent respiratory symptoms, caused by an abnormal inflammatory response to chronic inhalation of irritants. Chronic inflammation affects small airways and lung parenchyma, resulting in progressive airflow limitation (1). Environmental and host factors contribute to the pathogenesis and the heterogeneity of the disease (2, 3). Lately, the overlap between COPD and bronchiectasis is getting more attention as a potential COPD phenotype. Bronchiectasis is defined by permanently dilated and chronically inflamed middle-order bronchi, due to a vicious circle of transmural infection and inflammation. It translates into a clinical syndrome of productive cough, bacterial colonization and recurrent respiratory infections. The diagnosis of bronchiectasis is primarily based on imaging; with abnormal bronchial dilation on 1mm computed tomography (CT) images being the gold standard (4). Bronchodilation is assessed using Naidich's criteria: a broncho-arterial ratio >1, lack of tapering and the presence of bronchi within 1cm of costal pleura or abutting the mediastinal pleura (5).

The reported prevalence of bronchiectasis in COPD cohorts varies between 4 to 70%, due to differences in study population and radiological criteria (6). On the other hand, COPD is present in 3-35% of patients in bronchiectasis cohorts (7–9). Importantly, patients with COPD and bronchiectasis have a more severe disease course with higher mortality and an obvious need for adjusted therapeutic approaches (10–14). It is still unclear whether COPD facilitates bronchiectasis or if these two conditions co-exist in an individual patient.

Small airways with a luminal diameter <2mm are the major site of COPD pathogenesis and the main reason for airflow obstruction. It has been demonstrated that the numbers of small airways in COPD are reduced and that terminal bronchioles are narrowed in comparison with controls, even before the onset of emphysema (15, 16). More recently, a reduced total airway count on CT has been demonstrated in patients with mild COPD compared to never-smokers and smokers without COPD, suggesting that also large airways could play a role in the pathogenesis (17). In bronchiectasis, it is assumed that obliteration occurs in small airways, causing airflow obstruction (18, 19). So far, there are no advanced imaging studies in bronchiectasis focusing on airway generations beyond the resolution of conventional clinical

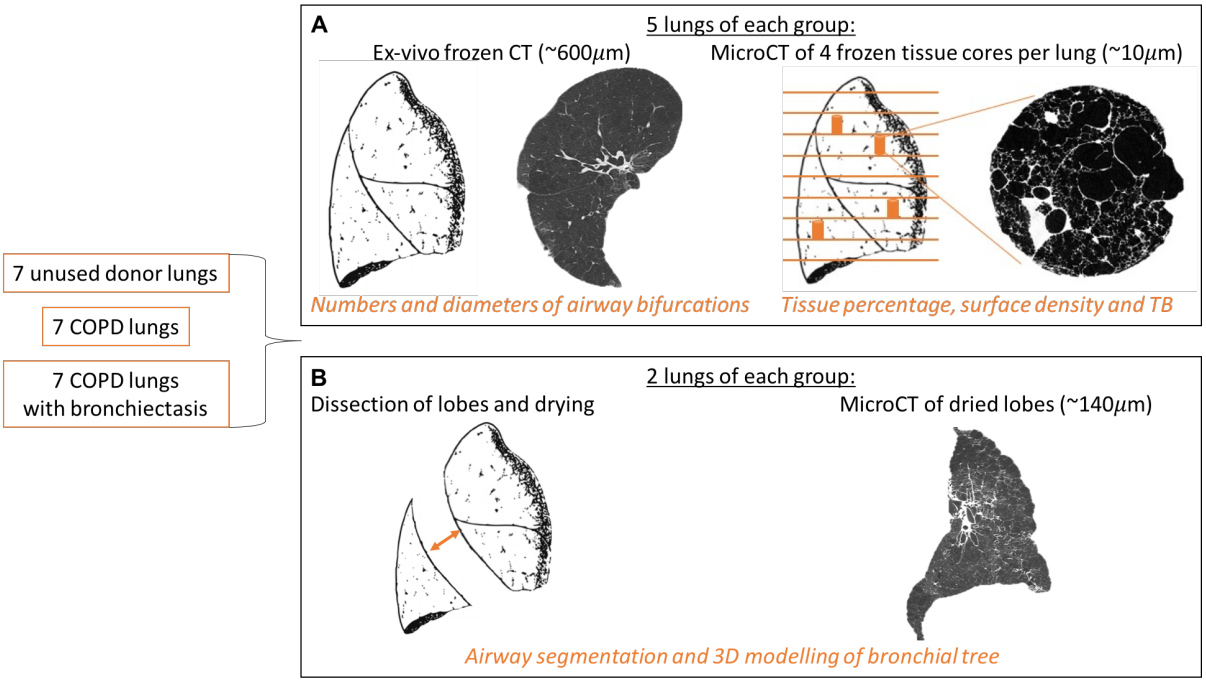
CT. MicroCT imaging, enabling evaluation up to the level of the most distal conducting airways or terminal bronchioles (16), is therefore a promising tool.

We provide for the first time a detailed morphometric analysis of the entire bronchial tree in COPD lungs with bronchiectasis in comparison to lungs of COPD patients without bronchiectasis and controls. In 14 COPD explant lungs and 7 unused donor lungs, we used a threefold imaging approach with high resolution CT (~600µm resolution), whole lobe microCT (~140µm resolution) and microCT scanning of randomly selected tissue cores (~10µm resolution) to investigate large and small airways up to the level of the terminal bronchioles.

MATERIALS AND METHODS

Study material

Figure 1: Overview of used materials and methods.



21 lungs were collected, comprised of 7 unused donor lungs, 7 COPD lungs and 7 COPD lungs with bronchiectasis. (A) Of each group, 5 lungs were prepared for freezing, CT scanning and further processed to obtain 4 tissue cores per lung for microCT. (B) Of two lungs per group, lobes were separated and air-dried to obtain microCT scans of the entire lobes. COPD: Chronic obstructive Pulmonary Disease, CT: computed tomography, TB: terminal bronchioles.

Explanted lungs of patients with end-stage COPD undergoing lung transplantation and of unused donors were prospectively collected in our center after approval of the local Medical Ethics Board of University Hospitals Leuven, Belgium (ML6385, s52174). Patients gave written informed consent to use their lungs for research purposes. According to Belgian law, declined organs from prospective donors can be used for research. Reasons for decline were: kidney tumor (n=1), recipient died (n=1), suspicion of beginning fibrosis in the contralateral lung (n=1), infection (n=1) and persistent micro-thrombi (n=3). The presence and morphology of bronchiectasis in COPD lungs was evaluated per lobe on clinical, pre-transplant inspiratory CT scans of the lungs with the modified Reiff score (lingula considered separately, tubular=1, varicose=2 or cystic=3 (20)) and confirmed by experienced chest radiologists (AD, JV). A thorough pre-transplant work-up could not reveal a specific cause for bronchiectasis. Based on availability and conservation, 5 COPD lungs with bronchiectasis, 5

COPD lungs without bronchiectasis and 5 unused donor lungs were retrospectively analysed (Figure 1A) and 2 lungs of each group were prospectively collected (Figure 1B). COPD lungs without bronchiectasis will be referred to as COPD lungs and unused donor lungs as controls hereafter.

Frozen whole-lung CT and tissue core microCT

15 explant lungs were inflated and frozen as previously described (16, 21). A CT scan was obtained from the frozen lungs, resulting in images with a resolution of approximately 600µm (Figure 1A). Emphysema was quantified as the percentage of lung area below -950HU with customized in-house software. An open-source image viewer, Horos software version 3.0 (Horosproject, www.horosproject.org) was used for measurements and manual counting of airway bifurcations per generation by scrolling through the ex-vivo whole-lung CT images. Inner diameters were measured manually at the widest level in each segment.

After CT scanning, two apical and two basal cores (2x1.4 cm) per lung were randomly obtained as previously described (16, 21). MicroCT scans (Bruker Skyscan 1172, Bruker, Kontich, Belgium) of the frozen tissue cores were performed (10µm resolution) (22) (Figure 1A). Tissue percentage and surface density of cores was calculated. Decreasing values of surface density reflect loss of normal tissue through disease (e.g. emphysema in COPD). Terminal bronchioles (TB), the last segments of conducting airways before alveolar buds become visible, were anatomically identified, counted and measured on the microCT images.

Air-dried whole-lobe microCT

2 lungs of each group were processed in order to obtain whole-lobe microCT images (Figure 1B). Lung lobes rather than whole lungs were scanned due to limitations on the volume of tissue that can be imaged within a single scan. Lobes were separated, cannulated and air-dried for 7 days while continuously inflating near total capacity. In case of right-sided lungs, the middle lobe was processed as part of the upper lobe. Lobes were scanned with a custom microCT set-up developed at the Ghent University Centre for X-ray Tomography (UGCT) (23), resulting in images with a resolution of approximately 140µm (Figure 1B). Additional detail on this set-up is provided in an online data supplement. Volume of the air-dried lobes and percentage of area below -950HU was obtained by StratX® Lung Analysis

Platform (Neuchâtel, Switzerland) on the in-vivo inspiratory CT scans. MicroCT images of the lung lobes were used for semi-automatic airway segmentation using itk-SNAP software (24) which resulted in 3D models of the bronchial tree. These models were used for automatic tracing and reconstruction in NeuronStudio software that allows calculation of airway numbers per generation and average inner diameter per segment (25).

Statistics

GraphPad Prism 7.0 (GraphPad, San Diego, CA, USA) was used to calculate differences between groups with Mann-Whitney or Kruskal-Wallis and Dunn's post-hoc test. The numbers of airways per generation were compared using 2-way ANOVA with Tukey's post-hoc test. Analysis of diameters with 2-way ANOVA was limited to the generations that were present in all lungs, and due to the processing of the lungs, diameters of generations 1 and 2 (1-3 in case of lobes), were not considered reliable. MicroCT results of tissue cores were analysed with mixed models using SAS 9.4 (SAS Institute Inc., Cary, NC, USA) with subject as random effect. P-values <0.05 were considered significant.

RESULTS

Numbers and diameters of airways on CT images

Characteristics of subjects of which the lungs were analyzed are presented in Table 1. All patients suffered from end-stage COPD. The subjects were not matched for sex. Bronchiectasis was present in at least 2 lobes per lung, none of the subjects had cystic bronchiectasis. Lung mass was significantly higher in the group with bronchiectasis compared to COPD (p=0.030) (Table 2). In terms of emphysema, COPD lungs showed a higher % of lung with a density below -950HU compared to control lungs (p<0.05), whereas no significant difference was found compared to COPD lungs with bronchiectasis (Table 2).

Table 1: Characteristics of patients of which an explanted lung was used for CT

	CONTROLS	COPD	COPD with BRONCHIECTASIS	*p*-value
Number	5	5	5	
Sex, M/F	4/1	1/4	5/0	**0.020**
Age, y	60 (52 - 69)	60 (56 - 62)	60 (54 - 61)	0.95
Height, cm	175 (164 - 178)	161 (157 - 169)	173 (173 - 179)*	**0.031**
BMI, kg/m^2	24.8	22.0	21.0	0.16
Pack years	NA†	30 (30 - 38)	39 (28 - 65)	0.55
FVC				
L	NA	1.83(1.66 - 2.07)	2.48 (2.02 - 3.47)	**0.032**
% pred	NA	65 (52 - 77)	61 (48 - 79)	1.00
FEV1				
L	NA	0.83 (0.60 - 0.86)	0.90 (0.62 - 1.18)	0.73
% pred	NA	30 (28 - 34)	22 (15 - 33)	0.31
DLCO, % pred	NA	34 (32 - 45)	28 (21 - 43)	0.42
Lobes with bronchiectasis	NA	0	2 (2 - 2.5)	
Modified Reiff score	NA	NA	5 (4-6)	

Results are presented as number or median (Q1-Q3). Gender was compared with Chi square. Age, height and BMI were compared by Kruskal-Wallis test, significant results found by Dunn's post hoc test are presented with symbols: * *p*<0.05 compared to COPD. Other variables were compared by Mann-Whitney test. COPD: Chronic Obstructive Pulmonary Disease, M: male, F: female, y: years, BMI: Body Mass Index, NA: not applicable, FVC: Forced Vital Capacity, FEV1: Forced Expiratory Volume in 1 second, DLCO: diffusion capacity for carbon monoxide. † these donors were registered as non-smokers at the time of allocation.

More generations were counted in lungs of COPD with bronchiectasis and controls compared to COPD (both p<0.05) (Table 2, Figure 2A). The number of bifurcations was higher in COPD with bronchiectasis and controls compared to COPD lungs (p<0.0001) (Figure 2A). No difference in bifurcation numbers was present between lungs with bronchiectasis and

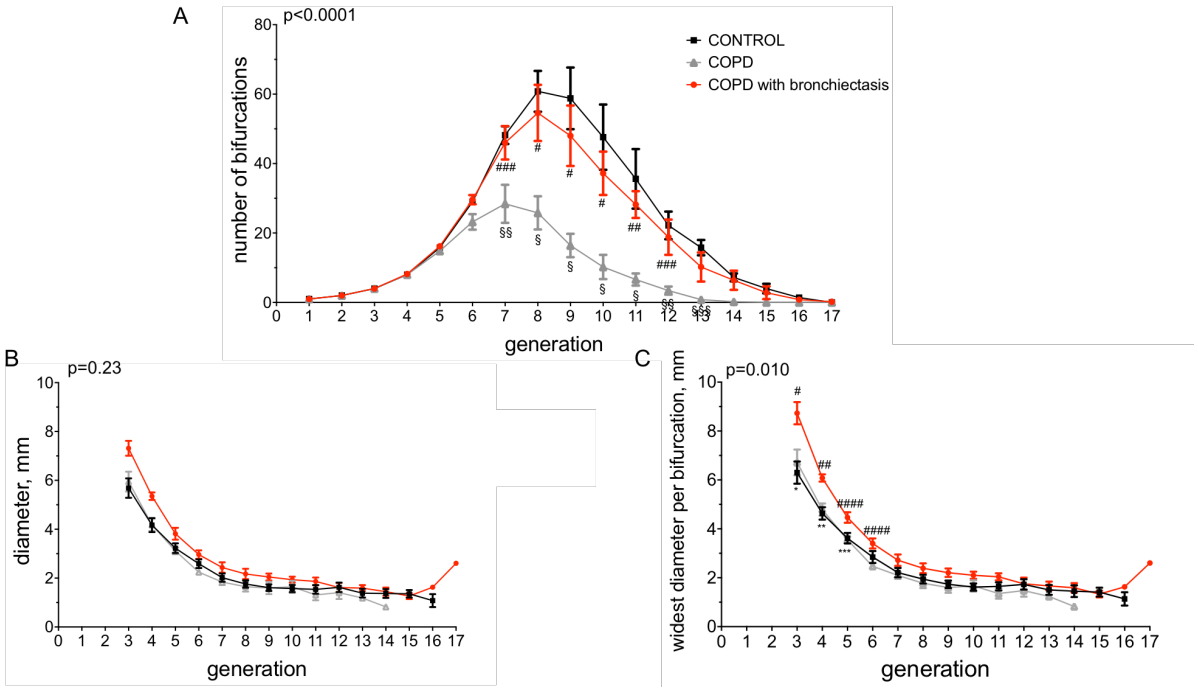
controls. Interestingly, the airway diameter was not different between the groups (p=0.23) (Figure 2B), unless the widest diameter per bifurcation was considered as a measure of lack of tapering (p=0.010) (Figure 2C).

Table 2: Results of frozen CT scans of the explanted lungs

	CONTROL	COPD	COPD with BRONCHIECTASIS	*p*-value
Left/Right lung	0/5	4/1	3/2	
Total lung volume, L	3.5 (2.7 - 3.6)	3.1 (2.6 - 3.4)	4.2 (3.5 - 5.0)	0.10
Lung mass, g	433 (316 - 486)	266 (227 - 300)	455 (370 - 561)**	**0.030**
Lung density, g/L	125 (117 - 134)	90 (78 - 99)	105 (82 - 151)	0.061
Area below -950HU, %	17.9 (11.2 - 22.1)	44.0 (35.4 - 49.4)*	31.2 (14.4 - 47.5)	**0.027**
Airway generations, n	16 (15 - 16.0)	12 (12 - 14)*	15 (15 - 16)**	**0.011**

Results are presented as median (Q1-Q3). *p*-values obtained by Kruskal-Wallis test. Significant results found by Dunn's post hoc test are presented with symbols: * $p<0.05$ compared to control, ** $p<0.05$ compared to COPD.

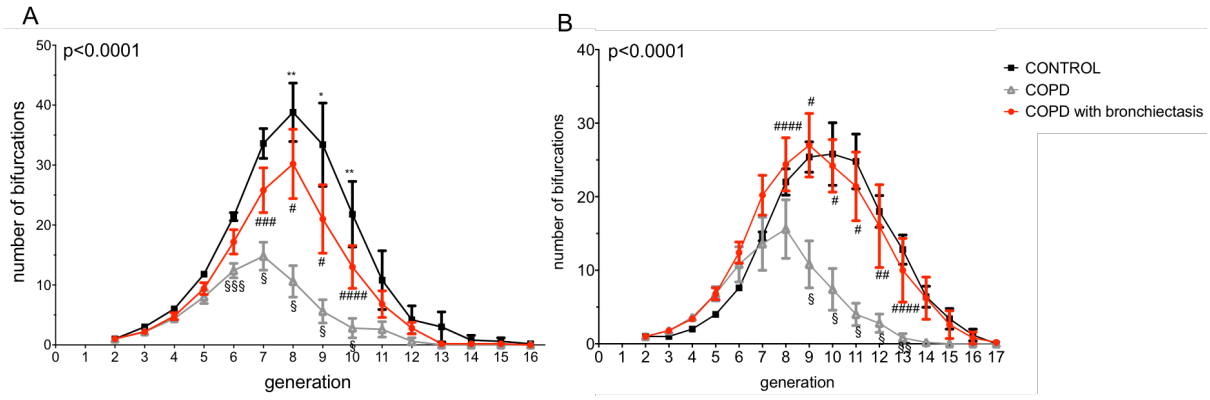
Figure 2: Bifurcation numbers and diameters on CT images of frozen lungs



Results of measurements on CT images of control lungs (n=5), COPD lungs (n=5) and COPD lungs with bronchiectasis (n=5). (A) Number of bifurcations per generation. (B) Diameters of airway segments per generation. (C) Widest diameter per bifurcation, lack of tapering. Results are presented as mean ± sem. Significant results of two-way ANOVA with Tukey's post-hoc test are presented with symbols. § p<0.0001, §§ p<0.001 and §§§ p<0.05 for COPD compared to control. * p<0.0001, ** p<0.001 and *** p<0.05 for COPD with bronchiectasis compared to control. $^{\#}$ p<0.0001, $^{\#\#}$ p<0.001, $^{\#\#\#}$ p<0.01 and $^{\#\#\#\#}$ p<0.05 for COPD with bronchiectasis compared to COPD.

Within the COPD lungs with bronchiectasis, only one showed bronchiectasis in the upper lobe. If upper and lower lobes were considered separately, the higher number of airways persisted in the group with bronchiectasis in comparison to COPD ($p<0.0001$), although more pronounced in the lower lobes (Figure 3). In comparison with control lungs, lungs with bronchiectasis showed less bifurcations from generation 8 to 10 in the upper lobes, whereas no differences were found in the lower lobes (Figure 3).

Figure 3: Number of bifurcations per lobe as counted on CT images of frozen lungs



Results of measurements on CT images per lobe of control lungs (n=5), COPD lungs (n=5) and COPD lungs with bronchiectasis (n=5). (A) Number of bifurcations per generation in the upper lobes (in case of right lung, middle lobe was considered part of the upper lobe). (B) Number of bifurcations per generation in the lower lobes. Results are presented as mean ± sem. Significant results of two-way ANOVA with Tukey's post-hoc test are presented with symbols. § $p<0.0001$, §§ $p<0.001$ and §§§ $p<0.05$ for COPD compared to control. * $p<0.01$ and ** $p<0.05$ for COPD with bronchiectasis compared to control. $^{\#}$ $p<0.0001$, $^{\#\#}$ $p<0.001$, $^{\#\#\#}$ $p<0.01$ and $^{\#\#\#\#}$ $p<0.05$ for COPD with bronchiectasis compared to COPD.

Numbers and diameters of airways on lobar microCT images

Characteristics of the patients of which lobar microCT was performed, are summarized in Table 3. Figure 4 shows airway segmentation on lower lobes of a control lung, a COPD lung and a COPD lung with bronchiectasis. The higher resolution of these microCT scans, in comparison to the previously described CT evaluation, is reflected by the higher number of generations counted on these images (eg 22 and 19 versus 16 generations in lower lobes of control lung) and enables us to evaluate smaller airways into more detail. The 3D models of all lobar bronchial trees are shown in Supplement Figure E1. The % of area -950HU was similar in the upper lobes of COPD lungs with and without bronchiectasis, in contrast to the lower lobes where the COPD lungs with bronchiectasis seem to demonstrate a lower percentage of emphysema (Table 3).

Table 3: Characteristics of subjects and results of airway segmentation on lobar microCT

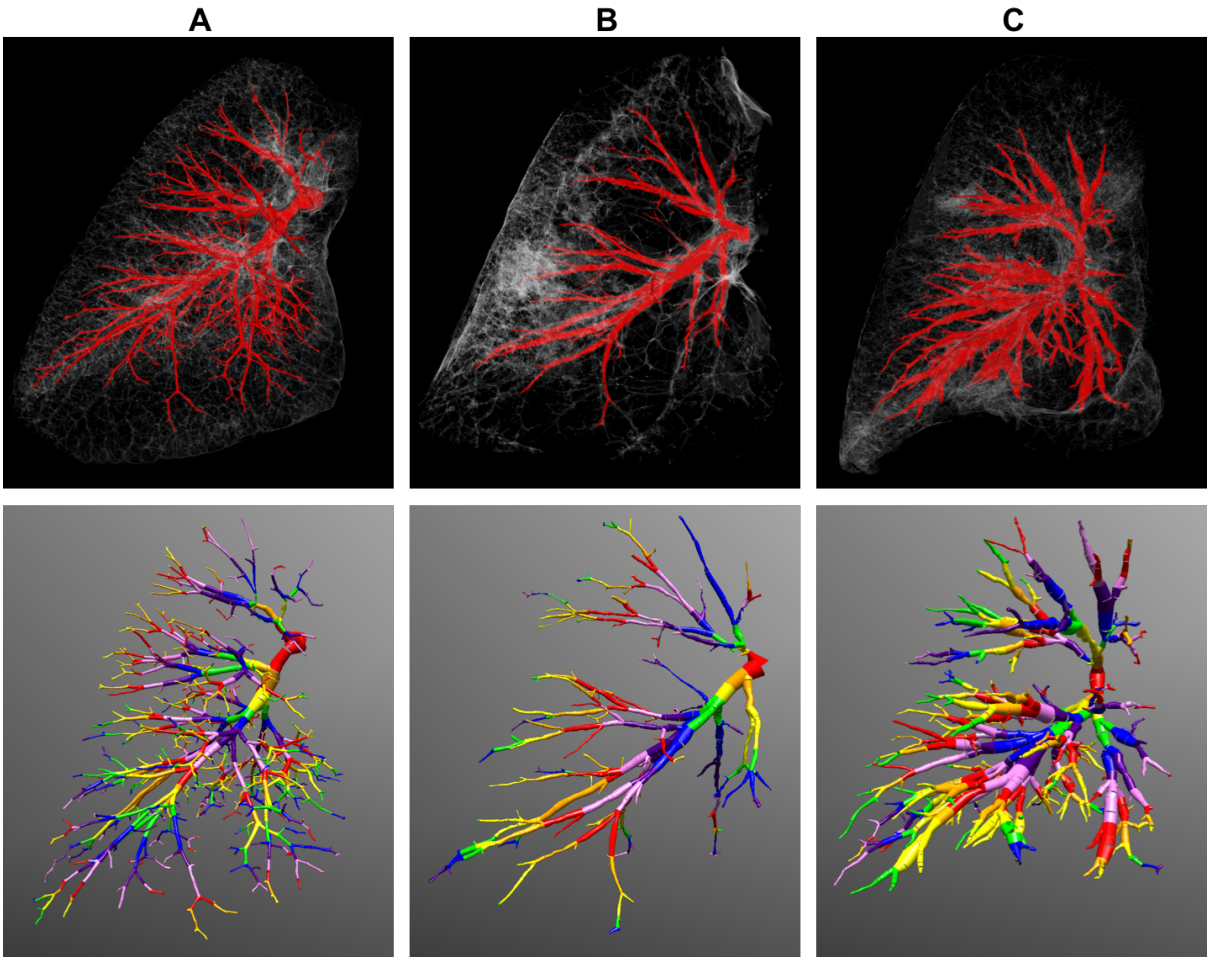
	CONTROL		COPD		COPD with BRONCHIECTASIS	
Sex, M/F	M	M	M	M	M	M
Age, y	58	57	64	64	61	65
Height, cm	185	180	170	168	173	170
Weight, kg	95	80	70	62	78	60
BMI, kg/m^2	27.8	24.7	24.2	22.0	26.0	20.8
Pack years	NA*	NA*	40	40	50	40
FVC						
L	NA	NA	3.18	1.65	2.48	3.70
% pred	NA	NA	84	45	61	97
FEV1						
L	NA	NA	0.82	0.88	0.48	1.38
% pred	NA	NA	28	30	15	46
DLCO, % pred	NA	NA	32	34	38	28
Lobes with bronchiectasis	NA	NA	0	0	2	3
Modified Reiff score	NA	NA	NA	NA	4	4
Right/left lung	Right	Left	Right	Left	Right	Left
UPPER LOBE†						
Volume, L	NA	NA	2.477	1.697	2.095	1.863
Area below -950HU, %	NA	NA	38	40	37	35
Generations, n	19	19	14	14	14	18
Airways, n	1478	958	324	224	602	628
LOWER LOBE						
Volume, L	NA	NA	1.625	1.317	1.329	0.847
Area below -950HU, %	NA	NA	33	38	24	23
Generations, n	22	19	19	17	20	17
Airways, n	1350	1129	364	384	683	926

Individual results are presented. COPD: Chronic Obstructive Pulmonary Disease, M: male, F: female, y: years, BMI: Body Mass Index, NA: not applicable, FVC: Forced Vital Capacity, FEV1: Forced Expiratory Volume in 1 second, DLCO: diffusion capacity for carbon monoxide. *These donors were registered as non-smokers at the time of allocation. †Sum of upper and middle lobe in case of right lung.

After segmentation, the COPD lungs with bronchiectasis showed considerably more airways from generation 9 to 12 in the upper lobes and generation 10 to 14 in the lower lobes, compared to COPD lobes (Table 3, Figure 4, Figure 5A and 5B). If these bronchiectatic lungs were compared with controls, lower airway numbers were present in generation 10 to 14 in upper lobes and generation 13 to 16 in lower lobes (Figure 5A and B). In COPD lobes, numbers of airways were reduced beyond generation 9 in upper lobes and beyond generation 10 in lower lobes when compared to controls (Table 3, Figure 5A and 5B). Throughout the first generations of the lower lobes, the diameters of COPD lungs with bronchiectasis were wider compared to COPD lungs (Figure 4, Figure 5D) and narrower in COPD lungs compared to control ($p=0.0098$). This difference was not present in the upper lobes (Figure 5C) ($p=0.30$). If the airways in the lower lobes were separated by diameter of

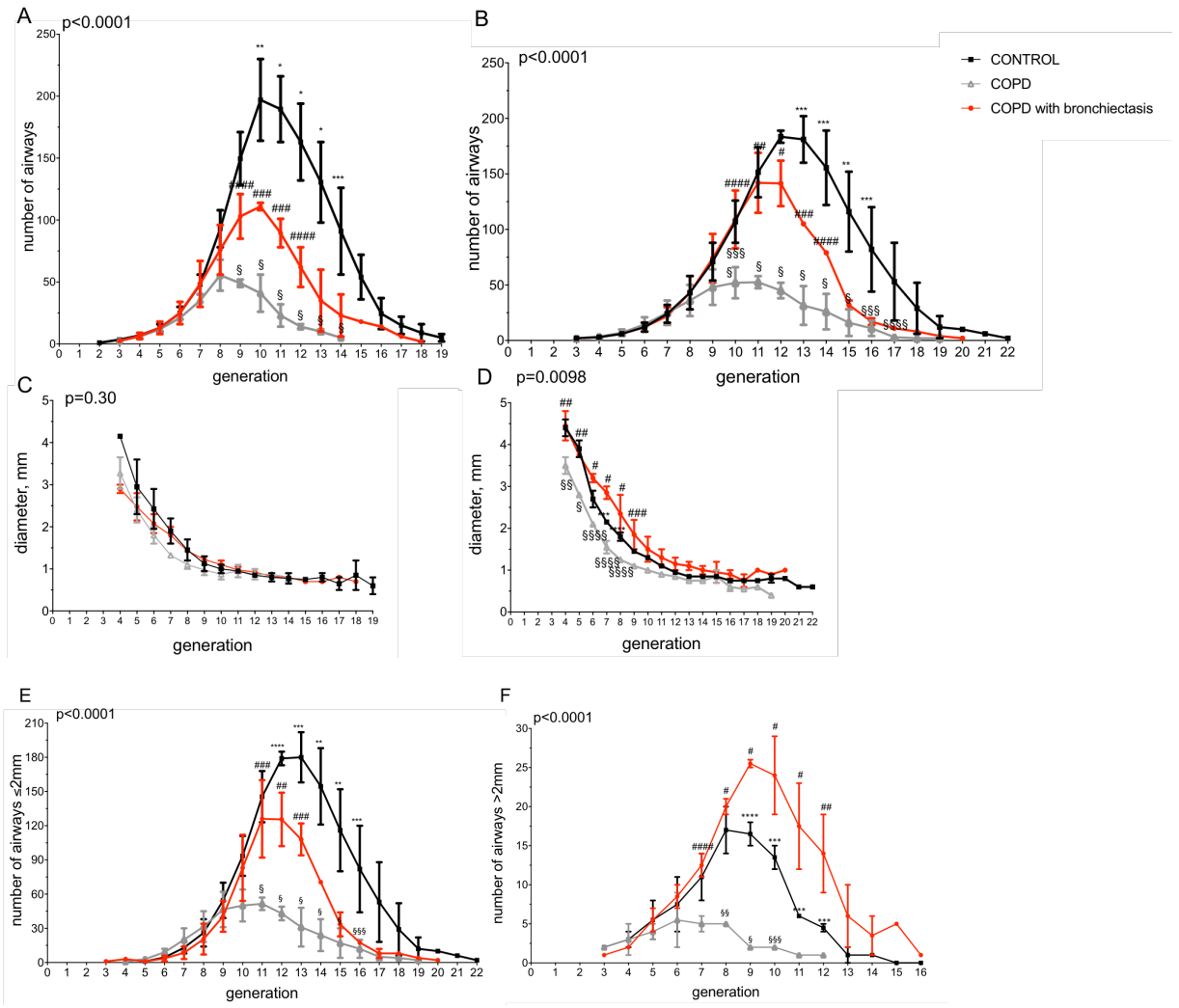
more or less than 2mm, the difference between COPD lungs with and without bronchiectasis was maintained, but particularly present in larger airways (>2mm), whereas the difference between COPD and control was more pronounced in the small airways (≤2mm) ($p<0.0001$) (Figure 5E and Figure 5F).

Figure 4: Airway segmentation of lower lobes on microCT images



Lower lobes of a (A) control lung, (B) COPD lung and (C) COPD lung with bronchiectasis. The upper panels show the bronchial tree after segmentation, projected on the contours of the lobe. The lower panels show the same bronchial tree after automated annotation with each color representing a different generation.

Figure 5: Airway numbers and diameters as calculated on segmented airway models obtained by microCT of air-dried lobe



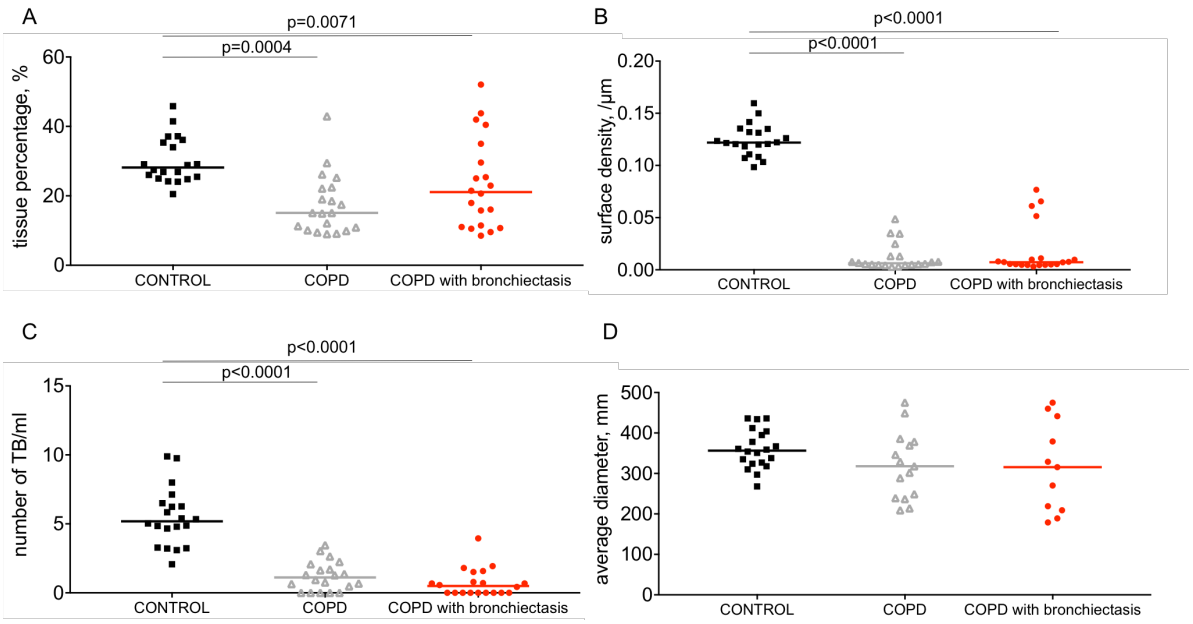
Results of measurements on microCT of control lobes (n=2 upper and n=2 lower lobes), COPD lobes (n=2 upper and n=2 lower lobes) and COPD lobes with bronchiectasis (n=2 upper and n=2 lower lobes). Graphs A and B show numbers of airways per generation and graphs C and D show average diameters of airway segments per generation in upper (A and C) and lower (B and D) lobes. In graphs E and F airways in the lower lobes were separated based on diameter ≤2mm and >2mm respectively. Results are presented as mean ± sem. Significant results of two-way ANOVA with Tukey's post-hoc test are presented with symbols. § p<0.0001, §§ p<0.001, §§§ p<0.01 COPD and §§§§ p<0.05 for COPD compared to control. * p<0.0001, ** p<0.001, *** p<0.01 and **** p<0.05 for COPD with bronchiectasis compared to control. $^{\#}$ p<0.0001, $^{\#\#}$ p<0.001 and $^{\#\#\#}$ p<0.01 for COPD with bronchiectasis compared to COPD.

Tissue characteristics and terminal bronchioles on microCT images of frozen tissue cores

Tissue percentage, surface density and number of TB per ml was significantly lower in both COPD groups compared to control (Figure 6A-C). No difference was found in tissue percentage and surface density in tissue cores of COPD patients with or without bronchiectasis. Moreover, the number and diameter of TB per ml was similar between these

groups (Figure 6A-D and Supplement Table E1). Taking spatial distribution into account, no difference was found in any of the groups between cores from upper versus lower lobes, neither between cores from regions with bronchiectasis and regions without bronchiectasis (Supplement Table E2).

Figure 6: MicroCT analysis of tissue cores



Results of measurements on images of tissue core microCT (n=4 per lung, 5 lungs per group) of control lungs, COPD lungs and COPD lungs with bronchiectasis.
(A) Tissue percentage, (B) surface density, (C) number of terminal bronchioles per ml and (D) average diameter of visible terminal bronchioles. *p*-values were obtained by mixed models applied on individual core data with lung as random effect, comparing with the control group. The same model was performed for comparison of each group to COPD with bronchiectasis, no significant difference was found between COPD without versus with bronchiectasis.

DISCUSSION

To our knowledge, this is the first study investigating and comparing the entire bronchial tree in COPD lungs with and without bronchiectasis and the first report on microCT of large lung specimens. Our morphometric study shows a significantly higher airway count in COPD lungs with bronchiectasis compared to COPD lungs without bronchiectasis. The observed difference was present for both large and small airways, although the terminal bronchioles were similarly decreased. The comparison with control lungs confirmed a reduced total airway count in COPD, up to the level of the terminal bronchioles.

The finding of more airways in COPD lungs with bronchiectasis compared to COPD lungs without bronchiectasis may suggest less severe emphysema or airway obstruction at the moment of transplantation (16, 26). Nevertheless, COPD subjects with or without bronchiectasis showed no difference in forced expiratory volume in 1 second (FEV1) or in diffusion capacity (DLCO). The % of area below -950HU, a threshold commonly used to quantify emphysema on CT, was not different between the groups and the surface density measured on microCT of tissue cores was also similar. Recently, lower total airway count (assessed till generation 10) in early COPD stages has also been shown independently of emphysema, suggesting that other factors may drive the reduction of airways (17). However, the sequence in which emphysema and bronchiectasis develop in patients with COPD is still unclear.

Surprisingly, the airway diameters showed no significant differences on CT, despite prior group selection based on bronchial dilatation on in-vivo CT. When taking the widest diameter per bifurcation into account, it was clear that larger diameters were present in the group with bronchiectasis, which may correspond to lack of tapering. An alternative explanation may be the reduced total airway count in COPD, resulting in shifts of generations and subsequently comparison of unequal generations. Since we do not know from which generations airways were exactly lost, we cannot adjust for this. In the lobar microCT study, airway diameters were significantly larger in the lower lobes of the bronchiectasis group compared to COPD, as also visualized on the bronchial models. Furthermore, when focusing on the larger airways (>2mm), the number of airways per generation was higher in bronchiectasis compared to COPD and control lungs. Increased number of visible airways on CT could be explained by

increased airway diameter, as previously postulated in cystic fibrosis (28). However, our data do not fully corroborate with this idea, as the number of airways in the lower lobes was significantly higher in bronchiectasis from generation 10 onwards, whereas diameters were mainly wider in the preceding generations. Furthermore, the increased airway count was not related to presence of bronchiectasis, since upper lobes -which were less or not affected by bronchiectasis- also showed this difference.

The role of small airways is recognized in the pathogenesis of both COPD and bronchiectasis (15, 16, 18, 19). Therefore, it was of great interest to investigate the terminal bronchioles in lungs with both entities. To our surprise, and despite the striking difference observed in the preceding large and small airways, the numbers and diameters of terminal bronchioles were equally reduced. Furthermore, measures of parenchymal destruction were similarly disturbed in COPD lungs with and without bronchiectasis. There is no clear explanation for the observed differences between COPD lungs with and without bronchiectasis, but an intruiging hypothesis could be that COPD patients with bronchiectasis have a different bronchial tree from early life onwards, which could predispose them to development of bronchiectasis and/or make them less prone to the loss of airways. Recently, the importance of early life events on lung structure and function is well accepted (29, 30). Variation in segmental airway branching (31) and reduced total airway count (17) have been suggested as risk factors for COPD. Our results contribute to the idea that the composition of the bronchial tree is relevant in development of respiratory disease and may determine why patients with COPD will or will not develop bronchiectasis.

The relevance of bronchiectasis in patients with COPD is under debate because diagnostic criteria may overestimate bronchodilation (32, 33) and higher broncho-arterial ratios have been attributed to reduced vessel size rather than increased bronchial diameters in smokers (34). Moreover, we acknowledge that data on bronchial wall thickness could have contributed to our results. Unfortunately, our approach did not allow for quantification of vessel dimensions and direct measurement of bronchial walls. Another limitation of our study is that we only addressed COPD lungs eligible for transplantation. The use of end-stage disease lungs prevents us from drawing conclusions about early disease stages. We are aware that

the lungs in the CT analysis were not matched for gender and side (left/right), but we tried to overcome this mismatch by confirming the results in the lobar microCT analysis in which lungs were matched for these characteristics. Finally, sample sizes are low, given the limited availability of unused donor lungs and of COPD lungs with bronchiectasis. Nevertheless, the pronounced visual differences in a limited sample size are striking and should lead to future studies focusing on associations between COPD, bronchiectasis, emphysema and initial airway counts in longitudinal cohorts.

In conclusion, our study shows a bronchial tree with more airway branches in explant COPD lungs with bronchiectasis compared to COPD lungs without bronchiectasis, despite similar emphysema and loss of terminal bronchioles. Our findings indicate that the intrinsic composition of the airway tree may predispose to the phenotype of COPD.

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SUPPLEMENT

Materials and methods: Air-dried whole-lobe microCT

These dried lobes were scanned with HECTOR, a custom designed microCT set-up developed at the Ghent University Centre for X-ray Tomography (UGCT) (23). The setup consisted of an open-type X-ray tube with directional target (X-RAY WorX, Garbsen, Germany) and a PerkinElmer amorphous-Si flat panel detector (2048x2048 pixels of 200^2 μm^2 each) with a CsI scintillator. The X-ray tube and flat panel detector were static, while the lobe was rotated over 360° and two thousand projection images were recorded. The tube was operated at 80keV and 200μA target current, and the reconstruction was performed with Octopus Reconstruction (XRE, Ghent, Belgium) (1) resulting in datasets of around 2000×2000×1500 voxels with a resolution of approximately 140µm (Figure 1B).

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Figure E1: Segmented airway models of all lobes

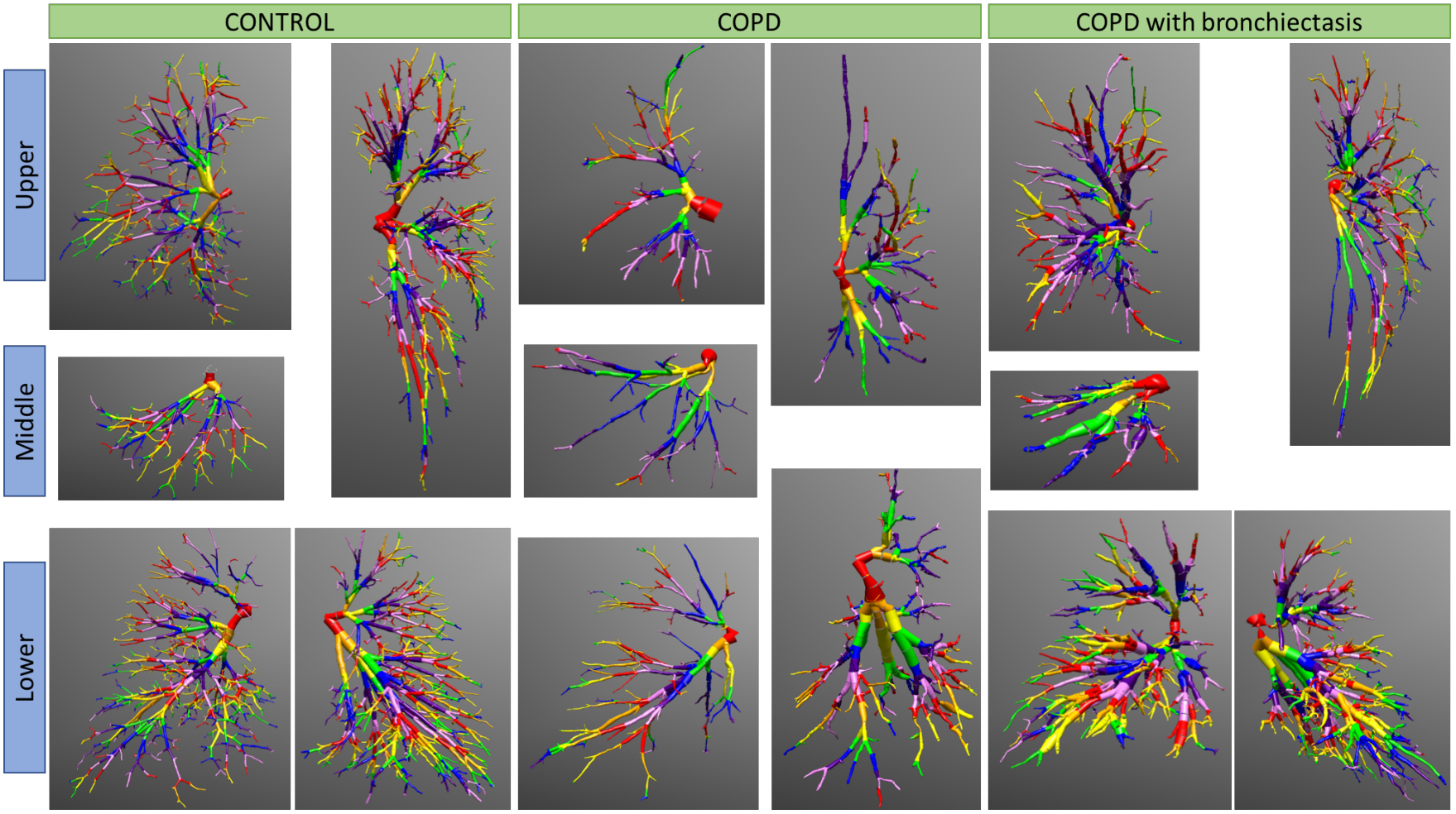

Table E1: Results of tissue core microCT measurements

	COPD LUNGS			COPD LUNGS with BRONCHIECTASIS		
	estimate	lower-upper limit	*p*-value	estimate	lower-upper limit	*p*-value
Tissue percentage, %	-11.32	-17.33 - -5.30	0.0004	-8.70	-14.94 - -2.47	0.0071
Surface density, /µm	-0.11	-0.12 - -0.098	<0.0001	-0.11	-0.12 - -0.10	<0.0001
Visible TB, n/ml	-4.14	-5.050 - -3.22	<0.0001	-4.99	-5.93 - -4.046	<0.0001
Diameter of TB, µm	-34.75	-87.70 - 18.20	0.19	-58.63	-118.75 - 1.49	0.056

Results are presented as estimates with upper and lower limits. *p*-values were obtained by mixed models applied on individual core data with lung as random effect, comparing with the control group. The same model was performed for comparison to COPD with bronchiectasis, no significant difference was found between COPD without versus with bronchiectasis.

Table E2: MicroCT results of upper versus lower lobes and bronchiectatic versus non-bronchiectatis regions

	Upper lobe	Lower lobe	*p*-value
CONTROL cores, n	15	5	
Tissue percentage, %	26.88 (24.99 - 35.35)	29.13 (25.78 - 41.46)	0.43
Surface density, /µm	0.12 (0.12 - 0.13)	0.12 (0.11 - 0.15)	0.93
TB/ml, n	5.028 (3.28 - 6.27)	5.84 (3.94 - 8.88)	0.60
COPD cores, n	10	10	
Tissue percentage, %	11.72 (9.80 - 18.66)	20.59 (13.73 - 26.33)	0.11
Surface density, /µm	0.0053 (0.0041 - 0.012)	0.0077 (0.0056 - 0.019)	0.11
TB/ml, n	0.38 (0 - 1.84)	1.33 (0.67 - 2.22)	0.18
COPD with bronchiectasis cores, n	8	12	
Tissue percentage, %	24.01 (20.87 - 49.54)	16.87 (10.82 - 32.58)	0.25
Surface density, /µm	0.0079 (0.0060 - 0.048)	0.0052 (0.0046 - 0.041)	0.20
TB/ml, n	0.28 (0 - 0.77)	0.56 (0 - 1.57)	0.78
	Non-bronchiectatic region	**Bronchiectatic region**	
COPD with bronchiectasis cores, n	10	10	
Tissue percentage, %	24.01 (11.27 - 45.85)	20.83 (10.66 - 27.77)	0.49
Surface density, /µm	0.0077 (0.0052 - 0.024)	0.0066 (0.0047 - 0.052)	0.68
TB/ml, n	0 (0 - 0.82)	0.097 (0.033 - 1.58)	0.088

Results are presented as n or median (Q1-Q3). P-values were obtained by Mann-Whitney test

Chapter 5

A diagnostic algorithm to prospectively differentiate asthma, ACO and COPD

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Preliminary data – work in progress

ABSTRACT

Background

The discrimination of obstructive airway diseases is not always straightforward, although a correct diagnosis is relevant for therapy. Asthma-COPD overlap (ACO) is poorly defined and specific tests or clinical criteria are missing.

Methods

Patients with suspicion of obstructive airway disease were included in this prospective study. A diagnostic algorithm was used to assign patients to 4 different groups with corresponding treatment. All subjects underwent measurement of lung function, diffusion capacity, fractional exhaled NO and blood analysis. Histamine provocation was performed in patients with non-obstructive post-bronchodilator FEV1/FVC, whereas subjects with obstructive FEV1/FVC underwent chest CT. Sputum induction was additional. Follow-up was scheduled at 3 and 12 months, a final expert diagnosis was assigned upon review of all clinical data.

Results

180 patients were included of which 160 were used for analyses. The algorithm could assign a diagnosis in 92% of subjects; asthma (n=50), obstructive asthma (n=17), ACO (n=13), COPD (n=56) and healthy control (n=11). Demographics and symptom scores were not different. Groups with obstructive post-bronchodilator FEV1/FVC showed no difference in spirometry and lung volumina, except for reversibility which was higher in ACO. The difference was mainly in the presence of emphysema (in COPD and ACO) and Th2 signature (in obstructive asthma and ACO). However, expert diagnosis deviated from the algorithm in 21% of cases. Evolution of diseases showed a trend towards better symptom scores in all groups, despite stable FEV1. Machine learning strategies confirmed the contribution of emphysema to the differential diagnosis, but was not able to create a better model for diagnosis of ACO.

Conclusion

A pre-defined diagnostic algorithm was able to assign diagnosis in the majority of patients with obstructive lung disease. This diagnosis was subject to change by the clinical evolution over time.

INTRODUCTION

In this era of pheno- and endotyping, the overlap of asthma and chronic obstructive pulmonary disease (COPD) or asthma-COPD overlap (ACO) gains a lot of interest. Although both are inflammatory airway diseases characterized by airflow limitation, asthma and COPD generally have a different nature of inflammation, onset, course and treatment. Nevertheless, symptoms of cough, breathlessness, wheezing and chest tightness are similar (1). The differential diagnosis of asthma and COPD is generally based on spirometry, namely on the respective presence or absence of reversible airway obstruction after inhalation of bronchodilators. However, it may be difficult to distinguish asthma from COPD in patients with an abnormal spirometry, since asthma may progress to persistent airflow obstruction, which is the hallmark of COPD (2,3). On the other hand, (partial) reversibility of airflow obstruction is often determined in COPD (4). Moreover, T helper type 2 (Th2) or eosinophilic inflammation, that was historically thought to be typical for asthma, is now recognized as a biomarker associated with exacerbations and better response to inhaled corticosteroids (ICS) in a subset of patients with COPD (5,6), whereas a neutrophilic phenotype is also recognized in asthma (7). Furthermore, it is not unlikely that COPD and asthma occur in the same individual, especially in subjects above 40 years of age with a history of considerable exposure to noxious stimuli. These patients, having features of both asthma and COPD, add to the complexity of diagnosing obstructive lung diseases. Despite efforts to better define ACO (8,9), no specific criteria or tests are validated for the diagnosis. Besides spirometry, different additional investigations are routinely performed in the diagnostic workup (8), but which feature contributes most in the differential diagnosis between asthma, COPD and ACO is not known.

The prevalence of ACO is dependent on the definition and the studied population (10,11). In COPD and asthma populations, the prevalence of overlap varies from below 10% to 50% and more (11,12). What most reports have in common, is an age-dependency, with increased chances of having ACO at higher ages (13). Although contrasting results are reported in terms of morbidity, progression and mortality in patients with ACO versus COPD, the majority of studies demonstrate more frequent or severe exacerbations and more pronounced FEV1

decline (14–16). Moreover, recognition of ACO has an impact on the therapeutic strategy, as administration of ICS, montelukast or even biologicals approved for treatment of severe asthma, may be considered. Likewise, one may prescribe tiotropium and azithromycin in asthma patients with persistent obstruction and/or neutrophilic inflammation (17,18). Unfortunately, hardly any randomized controlled trial has been performed to investigate pharmaceutical therapy in ACO, since asthma patients who smoked are generally excluded from asthma trials and COPD patients with a history of asthma are generally not included in COPD trials. Therefore, caution is necessary as the risk-benefit ratio of ICS and other drugs in ACO is not clarified yet.

As disease progression and management differ between asthma, COPD and ACO, a correct diagnosis of the obstructive airway disease is necessary. In order to offer personalized and evidence-based treatment to our patients with ACO, we urgently need a consistent approach to guide us in designing studies that include these "real-world" patients.

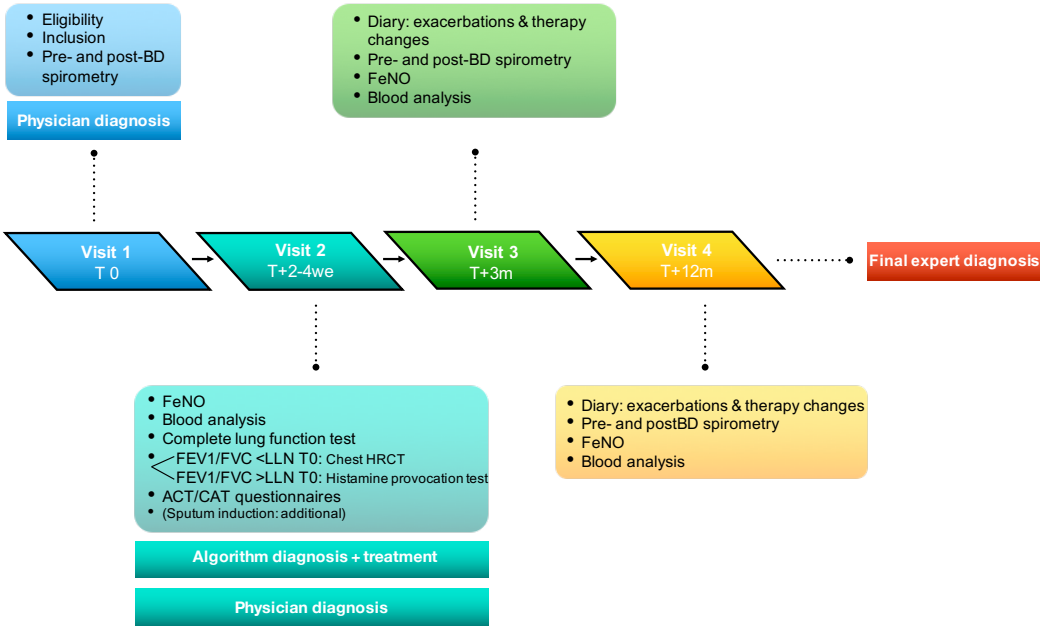
This prospective study aimed to test a diagnostic algorithm for obstructive airways diseases, that may be used in daily practice, to obtain a correct diagnosis and, hence, initiate an adequate therapy according to the current guidelines. In a next step, the study evaluated which different tests contributed most to the diagnosis with the help of supervised machine learning.

MATERIALS AND METHODS

Study design and subjects

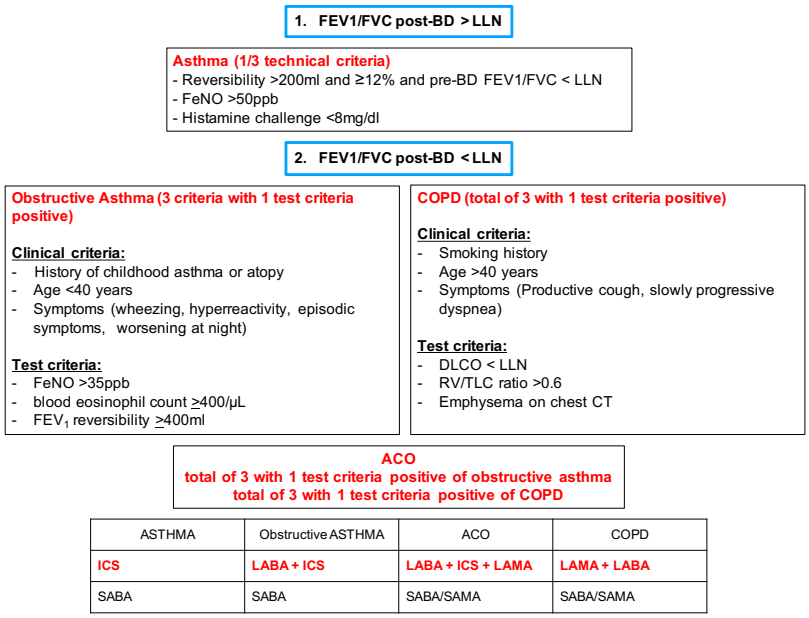
In this prospective, non-randomized single center study, patients were included in the outpatient respiratory department between June 2015 and June 2017. Inclusion criteria were Caucasian descent, age 30-80 years and suspicion of obstructive airway disease. The latter criterion was based on presence of symptoms (cough, sputum production, dyspnea or wheezing) or history of asthma, COPD or use of inhalation therapy in primary care. Exclusion criteria were the need for hospitalization, treatment with systemic corticosteroids or long-term antibiotics, pregnancy, clinically significant cardiovascular disease and history of other, non-obstructive pulmonary disease. Figure 1 shows timeline and design of the study. Eligible subjects were assigned to a diagnostic algorithm based on a post-bronchodilator spirometry at the first visit (Figure 2). The algorithm was developed based on current literature and guidelines (8,19) and integrates tests that are routinely performed in this patient population, next to blood sampling. Every subject was proposed to perform a sputum induction as an additional test. Inhalation therapy was initiated based on the algorithm diagnosis and in agreement with the current standard of care, molecule and device according to choice (Figure 2)(8,19). Treating physicians were asked to reveal their diagnosis at visit 1 (before diagnostic workup) and visit 2 (after diagnostic workup). This physician's diagnosis at visit 2 was expressed as agreement with the algorithm diagnosis on a visual analogue scale (VAS) and the suggestion of an alternative diagnosis if not agreed upon. Subjects were expected to fill in a monthly diary, to report exacerbations and/or changes in therapy. Follow-up was scheduled after 3 and 12 months. A "final expert diagnosis" was assigned by a panel of 4 experienced pulmonologists upon review of all clinical data, after visit 4 or after the last visit before drop-out. All patients provided written informed consent (with or without permission for sputum induction) and the study was approved by the local Ethics Committee (Medical Ethical Board of the University Hospitals Leuven, Belgium – B322201524606).

Figure 1: Study design and time line



Study timeline from inclusion (visit 1) till end of study after 12 months (visit 4). Diagnoses were assigned on 3 occasions: by physician on visit 1 and 2, by the study algorithm on visit 2 and by an expert panel upon review of all data after visit 4 or sooner if the subject dropped-out.

Figure 2: Diagnostic algorithm



The diagnostic algorithm as used in the study protocol to assign patients to 4 groups; asthma, obstructive asthma, ACO and COPD. Based on these labels, a therapeutic strategy was applied.

Pulmonary function tests and questionnaires

All pulmonary function tests were performed according to the American Thoracic Society and European Respiratory Society (ATS/ERS) standard (20) using standardized equipment (Sensor- medics Whole Body Plethysmograph; Viasys Healthcare, Vilvoorde, Belgium). Lung volumes were measured with a constant volume body plethysmograph, at a breathing frequency of 35-40 breaths per min. Diffusion capacity of the lung for carbon monoxide (DLCO) was measured by the breath-holding carbon monoxide gas transfer method (21). Predictive values and lower limits of normal (LLN) were calculated based on the reference equations proposed by the European Community for Steel and Coal in 1993 (35). Fractional exhaled nitric oxide (FeNO) analysis was performed by a nitric oxide chemiluminescence analyzer (Eco Medics CLD88sp, Duernten, Switzerland) with online measurement of FeNO following the 2005 ATS/ERS recommendations (23). Histamine provocation testing was performed in patients that demonstrated a non-obstructive post-bronchodilator (BD) forced expiratory volume in 1 second (FEV1)/forced vital capacity (FVC), by inhaled histamine starting at a concentration of 0.25mg/mL and doubling this concentration until FEV1 falls below 80 percent of the initial value or until a concentration of 8mg/mL was reached (24). Sputum was induced after pretreatment with 400µg of inhaled salbutamol, by inhalation of subsequent concentrations of hypertonic saline vapors (3%, 4% and 5%) generated by an Ultra-Neb ultrasonic nebulizer (DeVilbiss, Port Washington, NY, USA). Every concentration was inhaled for 5 minutes, where after sputum could be expectorated. The patient was asked to rinse the mouth thoroughly with water and spit the sputum into a collection tube (25). Both COPD Assessment Test (CAT™) (26) and Asthma Control Test (ACT) (27) were completed by all subjects at inclusion, visit 3 and visit 4. The higher the CAT score and the lower the ACT score, the worse the symptoms.

Blood and sputum analysis

Blood samples were collected at visit 2, 3 and 4. Complete blood count with differentiation of white blood cells was performed by fluorescence flow cytometry (Sysmex XE-5000, Kobe, Japan). Total IgE and specific IgE against the most frequent aero-allergens (house dust mite (*Dermatophagoides pteronyssinus*), grass pollen mix, birch pollen, mugwort, fungal mix and animal epitheles mix) were determined on visit 2 by ImmunoCAP

fluoroenzyme-immunoassay (Phadia AB, Uppsala, Sweden). Specific IgE values of 0.35kU/L were considered positive.

A differential cell count was performed on the induced sputum samples. After selection of sputum plugs, samples were homogenized, filtered and centrifuged. The resulting pellet was dissolved and cells were counted by microscope. Samples of 100µL were plated, plates dried, fixated and stained with May-Grünwald Giemsa. Differential cell count using a microscope, was performed if ≥400 non-squamous cells were present.

High resolution computed tomography (HRCT) of the chest

Subjects with obstructive FEV1/FVC after bronchodilation underwent HRCT of the chest with in- and expiratory images, according a 120kv protocol with 1mm slice-thickness and 0.6-0.7 collimation.

Statistics

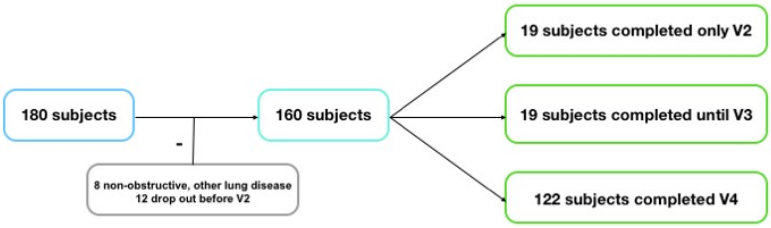
Analyses were performed with SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) and GraphPad Prism 7 (GraphPad, San Diego, CA, USA). Differences in proportions were calculated with chi^2 test and differences in median values with Kruskal-Wallis test and Dunn's post-hoc testing. The supervised machine learning strategy was performed in R version 3.3.1 (Boston, USA) with random forest models (randomForest package) using mean accuracy decrease and correlograms (corrplot package) to shortlist the variables that contributed most. Subsequently step-wise build-up and five-fold cross validation (caret package) was used to create a new model and calculate accuracies. T-distributed stochastic neighbor embedding (tSNE) plot (Rtsne package) was used to visualize clusters. P-values <0.05 were considered significant.

RESULTS

Group characteristics

180 patients were included. 12 patients dropped out before visit 2 and 8 patients were excluded because of arguments for other, non-obstructive lung disease (Figure 3). The algorithm was unable to assign 24 out of 160 subjects to a specific group, of which 11 were considered as healthy controls, since all investigations were negative, and 13 with an obstructive lung function were considered "undefined" (8%). Differences were investigated in 5 groups; asthma (n=50), obstructive asthma (n=17), ACO (n=13), COPD (n=56) and control (n=11).

Figure 3: Schematic representation of inclusions and drop-outs during the study protocol



There was no difference in demographic characteristics (Table 1). COPD patients smoked significantly more pack-years compared to asthma, obstructive asthma and controls, although the current smoking behavior was not different between groups. In terms of symptoms, ACT showed no overall difference nor in between groups, whereas CAT scores demonstrate a trend towards higher scores in ACO and COPD compared to the other groups. If specific symptoms were evaluated separately, only the proportion of patients presenting with dyspnea and hyperreactivity showed an overall significant difference (Figure 4A). Not surprisingly, a history of asthma or atopy was significantly different between groups and mainly present in obstructive asthma and ACO, although presence of childhood asthma was more equally distributed between groups (Figure 4B).

Table 1: Characteristics of algorithm-defined groups

	ASTHMA	Obstructive asthma	ACO	COPD	Control	p-value
Number	50	17	13	56	11	NA
Age, y	62 (51-67)	63 (46-69)	63 (55-71)	64 (59-69)	56 (50-69)	0.3198
Age >40y, %	92.0	88.2	100	96.4	90.9	0.5749
Sex, % male	44.0	76.5	61.5	55.4	36.4	0.1300
BMI, kg/m²	28 (24-31)	27 (25-30)	26 (23-31)	25.0 (21-28)	26 (25-30)	0.0997
Pack-years	16 (0-35)[#]	15 (0-26)[#]	30 (23-42)	40 (29-46)	10 (0-36)[#]	**<0.0001**
Current smokers, %	20.0	17.7	23.1	35.7	27.3	0.3684
Ever smokers, %	70.0	64.7	100	98.2	63.4	**<0.0001**
ACT	18 (14-20)	16 (11-19.5)	16 (14-18.5)	16 (13-18)	19 (13-20)	0.2427
CAT	14 (9-20)	15 (9-22.5)	19 (12-20)	18 (13-22)	12 (10-15)	0.0537
Pre-BD FEV1, % pred	87 (78-104)[#]	61 (58-77)[†§]	61 (52-71)[§]	56 (39-69)[†§]	104 (93-115)	**<0.0001**
Pre-BD FEV1/FVC, %	71 (65-79)[#]	55 (50-60)[†§]	55 (42-61)[§]	47 (39-58)[†§]	78 (74-80)	**<0.0001**
Post-BD FEV1, % pred	94 (81-105)[#]	72 (66-80)[†§]	73 (66-79)[§]	58(44-73)[†§]	106 (98-119)	**<0.0001**
Post-BD FEV1/FVC, %	73 (69-80)[#]	55 (54-63)[†§]	56 (47-61)[§]	48 (40-59)[†§]	77 (75-82)	**<0.0001**
Reversibility*, %	6	17.7	46.2	19.6	0	**0.0038**
Reversibility ≥400ml, %	2	17.7	38.5	1.8	0	**<0.0001**
RV/TLC	0.4 (0.3-0.5)[#]	0.4 (0.4-0.5)	0.5 (0.4-0.6)	0.5 (0.4-0.6)	0.4 (0.3-0.4)[#]	**<0.0001**
RV/TLC >0.6, %	4	0	15.4	23.2	9.1	**0.0172**
DLCO < LLN, %	48	5.9	69.2	85.7	18.2	**<0.0001**

Results are presented as n, % or median (Q1-Q3). Differences in proportions were analyzed by Chi square test, differences in medians by Kruskal-Wallis test with Dunn's post-hoc test. *Reversibility was defined as FEV1 change of ≥12% and >200ml after bronchodilation, [#]significantly different compared to COPD, [†]significantly different compared to asthma, [§]significantly different compared to control. ACO: Asthma-COPD Overlap, COPD: Chronic Obstructive Pulmonary Disease, y: years, BMI: Body Mass Index, ACT: Asthma Control Test, CAT: COPD Assessment Test, BD: bronchodilator, FEV1: Forced expiratory volume in 1 second, FVC: Forced vital capacity, RV: Residual volume, TLC: Total lung capacity, DLCO: Diffusion capacity of the lung for carbon monoxide, LLN: Lower limit of normal.

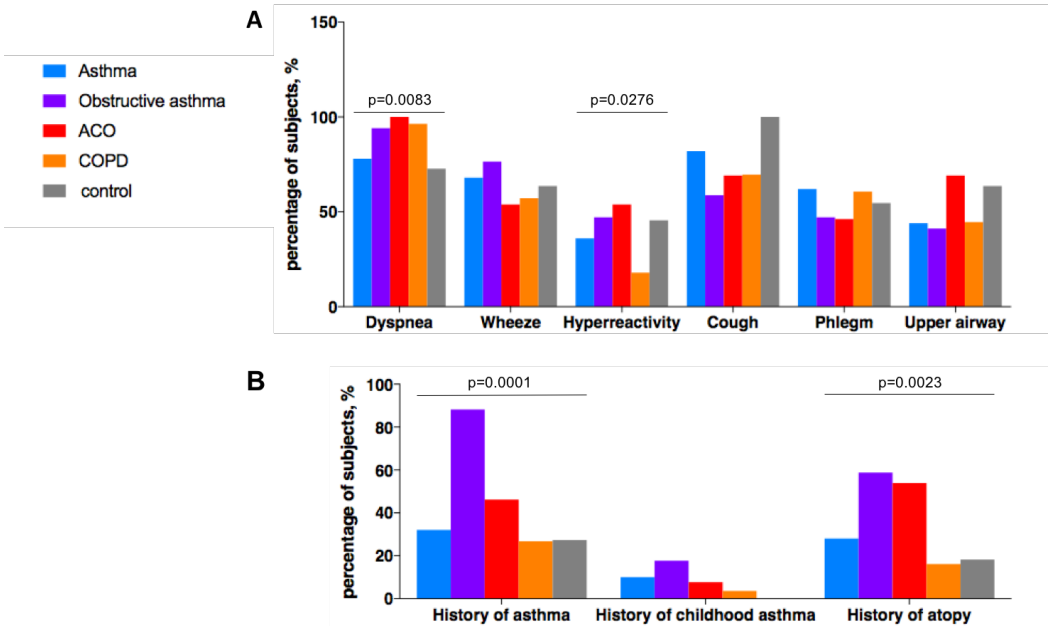
Both pre- and post-BD % predicted of FEV1 and FEV1/FVC were significantly lower in the obstructive diseases versus the non-obstructive groups. No difference in terms of FEV1 or FEV1/FVC was found between obstructive asthma, ACO and COPD. With regards to reversibility, the difference between groups was significant with mainly the patients assigned to the ACO group showing this feature.

The median residual volume (RV)/total lung capacity (TLC) ratio, as a measure of hyperinflation, was only higher in COPD versus asthma and control. On the other hand, the proportion of patients with a RV/TLC ratio >0.6 was 0% in obstructive asthma versus 15.4% and 23.2% in ACO and COPD, respectively.

Median % predicted of DLCO was significantly lower in COPD versus asthma, obstructive asthma and control. The ACO group demonstrated a lower median DLCO value compared to obstructive asthma (Figure 5A). Based on LLN, a considerable proportion of patients with asthma and even controls showed DLCO values < LLN (Table 1). If presence of emphysema on chest CT was evaluated in the subgroup with obstructive post-BD spirometry, 12.5% of obstructive asthma patients fulfilled this criterion versus 46% of ACO patients and 82% of COPD patients, which is significantly different between groups (Figure 5B).

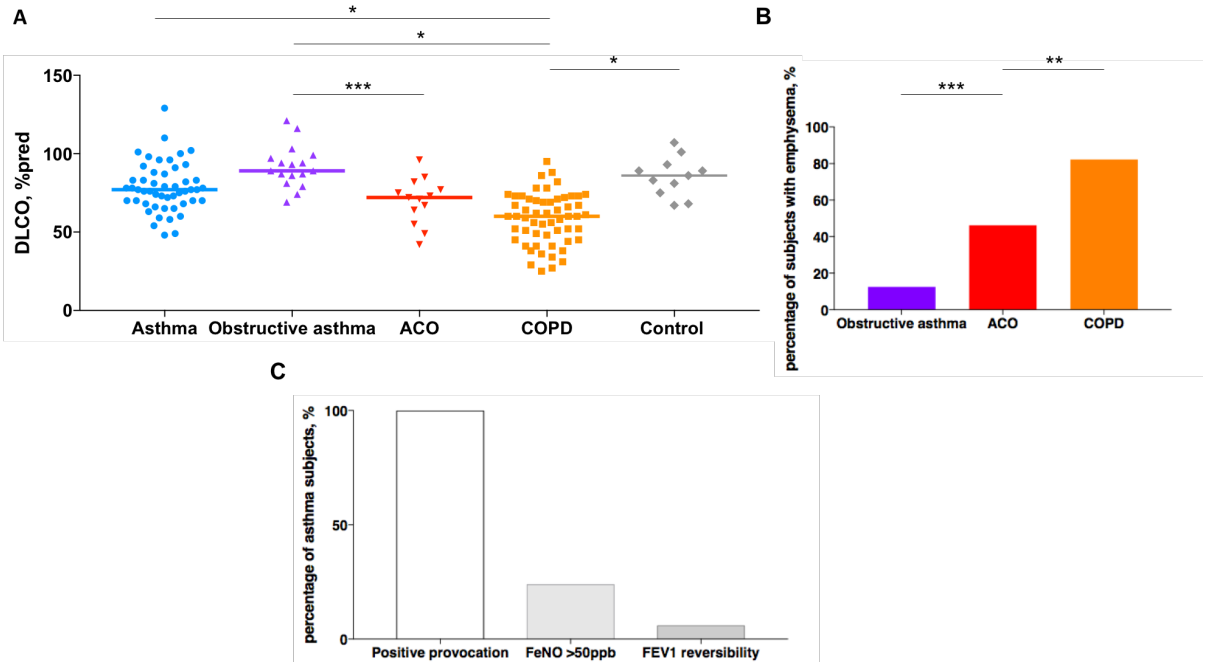
The histamine provocation test, which was only performed in subjects without obstructive post-BD spirometry, was positive in all asthma patients in contrast to none of the control subjects. Obviously, this criterion contributed most to the diagnosis of asthma in this study population (Figure 5C).

Figure 4: Specific symptoms and history per algorithm-defined group



Proportion of subjects presenting with specific symptoms (A) and history (B). Differences between groups were analyzed with Chi-square test.

Figure 5: Diffusion capacity, emphysema and asthma criteria



(A) Results of diffusion capacity measurement (% predicted) in algorithm-defined groups. Every colored symbol represents a subject and colored horizontal lines represent median values. Black lines represent significant differences obtained by Kruskal-Wallis test with Dunn's post-hoc test with *p<0.0001 and ***p<0.05. (B) Proportion of subjects with emphysema on chest CT in the subgroup with obstructive post-BD FEV1/FVC. Black lines represent significant differences obtained by Chi square test with **p<0.01 and ***p<0.05. (C) Fulfillment of criteria in the algorithm-defined asthma group. Reversibility was defined as FEV1 change of ≥12% and >200ml after bronchodilation.

Th2 signature

All features of Th2 inflammation were significantly different between groups (Table 2). Both continuous and proportional FeNO values were significantly higher in obstructive asthma and ACO compared to COPD (Figure 6A). In case of ACO, this eosinophilic airway inflammation was reflected by peripheral blood eosinophilia with significant higher values compared to asthma and COPD (Figure 6B). If different cut-offs for absolute eosinophil counts in blood were compared, no difference in proportion of patients with values ≥200/µL was present between groups, in contrast to a used cut-off of ≥300 or ≥400/µL (Table 2). Total IgE was only higher in obstructive asthma compared to controls, whereas the proportion of subjects sensitized against at least one of the frequent aero-allergens that were tested, was clearly higher in obstructive asthma, ACO and asthma (in descending order) compared to COPD and controls.

Table 2: Features related to Th2 inflammation per algorithm-defined group

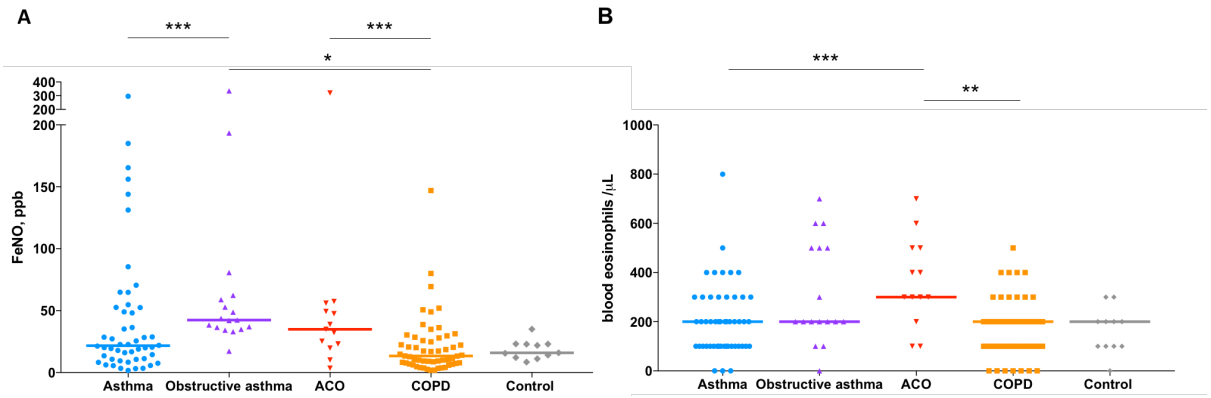
	ASTHMA	Obstructive asthma	ACO	COPD	Control	p-value
FeNO >35ppb, %	32	76.5	46.2	12.5	0	**<0.0001**
FeNO >50ppb, %	24	35.3	23.1	7.1	0	**0.0158**
Blood eosinophils, %	2.4 (1.4-3.6)	3.3 (2.2-5.9)	4.2 (3.5-5.5)	1.8 (1.0-3.1)	2 (1.4-3.4)	**0.0001**
Blood eosinophils ≥200/µL, %	58	82.4	84.6	53.6	54.6	0.0916
Blood eosinophils ≥300/µL, %	30	41.2	76.9	19.6	18.2	**0.0012**
Blood eosinophils ≥400/µL, %	14	35.3	46.2	8.9	0	**0.0015**
Total IgE, kU/L	107 (23-269)	143 (60-962)§	115 (27-463)	55.5 (22-196)	22 (8-62)	**0.0440**
Sensitized*, %	38	64.7	46.2	17.9	18.2	**0.0027**
Sputum induction, n	33	12	13	29	4	
Sputum eosinophils ≥3%, %	45.5	91.7	69.2	37.9	0	**0.0028**
Sputum neutrophils ≥76%, %	18.2	33.3	15.4	65.5	0	**0.0004**
Mixed granulocytic, %	3	25	0	17.2	0	0.0767
Paucigranulocytic, %	39.4	0	15.4	13.8	100	**0.0002**

Results are presented as n, % or median (Q1-Q3). Differences in proportions were analyzed by Chi square test, differences in medians by Kruskal-Wallis test with Dunn's post-hoc test. *A subject was considered sensitized if specific IgE was present against at least one of the tested allergens (house dust mite, grass pollen mix, mugwort, birch pollen, fungal mix or epitheles mix), §significantly different compared to control.

Sputum cell counts

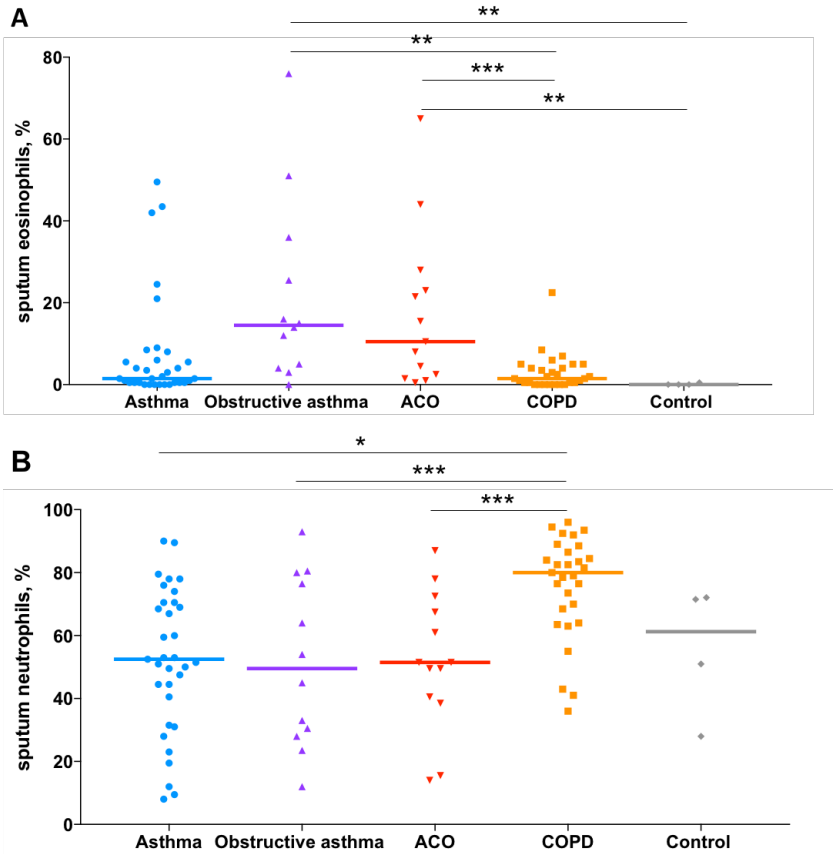
Differential cell count on induced sputum samples was an additional test, performed in less patients per group (Table 2). Nevertheless, results confirm the algorithm by showing significantly more sputum eosinophilia in both obstructive asthma and ACO compared to COPD, whereas sputum neutrophils were significantly more present in COPD versus asthma, obstructive asthma and ACO (Figure 7). This was confirmed if cut-offs for sputum eosinophilia and neutrophilia were used (28). None of the ACO subjects had a mixed inflammatory pattern. (Table 2)

Figure 6: FeNO and blood eosinophils per algorithm-defined group



Results of eosinophilic airway inflammation measured by FeNO (A) and blood eosinophils (B) per algorithm-defined group. Every colored symbol represents a subject and colored horizontal lines represent median values. Black lines represent significant differences obtained by Kruskal-Wallis test with Dunn's post-hoc test with *p<0.0001, **p<0.01 and ***p<0.05.

Figure 7: Differential cell counts on induced sputum samples per algorithm-defined group



Results of eosinophil (A) and neutrophil (B) counts on induced sputum samples. Every colored symbol represents a subject and colored horizontal lines represent median values. Black lines represent significant differences obtained by Kruskal-Wallis test with Dunn's post-hoc test with *p<0.0001, **p<0.01 and *** p<0.05.

Comparison of algorithm with physician's and expert diagnosis

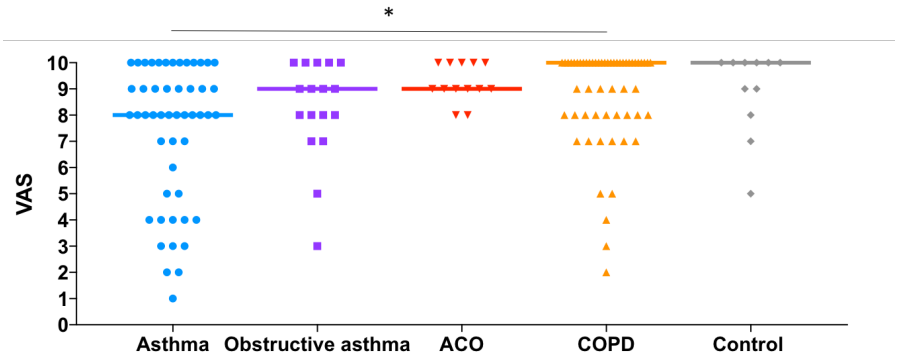
If algorithm diagnosis was compared to physician's diagnosis on visit 1 (Figure 1), the agreement was poor (Table 3). Comparison of physician's diagnosis on visit 1 and on visit 2, the latter based on results of all investigations except sputum induction for which the physician was blinded, shows an agreement <70%, illustrating the poor discriminating potential of symptoms and pre/post-BD spirometry without additional testing. Agreement between algorithm and physician's diagnosis on V2 is high, but may be biased by the fact that the physician was not blinded to the algorithm. Median agreement in terms of VAS was 9 (8-10) with significantly better agreement in COPD compared to asthma (Figure 8). Expert diagnosis showed an overall agreement of 78.9% with the algorithm (Table 3). Disagreement in favor of ICS use is presented in Table 3.

Table 3: Agreement between algorithm, physician's and expert diagnoses

	Asthma	Obstructive asthma	ACO	COPD	Control	Overall agreement	ICS disagreement
Algorithm vs diagnosis V1	62%	11.8%	100%	67.9%	9.1%	57.8%	17.7% *
Diagnosis V1 vs V2	70.5%	57.1%	39.3%	80.4%	50%	67.3%	NA
Algorithm vs diagnosis V2	80%	88.2%	100%	87.5%	90.9%	86.4%	4.8% *
Diagnosis V2 vs expert diagnosis	95%	40%	55.6%	87%	80%	75.6%	6.8% #
Algorithm vs expert diagnosis	80%	47.1%	84.6%	85.7%	81.8%	78.9%	3.3% #

Agreement between different diagnoses, expressed as %. V1: visit 1, based on history and pre/post bronchodilator spirometry, V2: visit 2, based on results of all performed investigations except differential cell count on sputum, expert diagnosis: final diagnosis assigned by a panel of 4 experienced pulmonologists upon review of all clinical data except sputum, and blinded for other diagnoses. Disagreement in favor of ICS use *compared to the algorithm diagnosis, and # compared to the expert diagnosis.

Figure 8: Agreement of physician with algorithm diagnosis

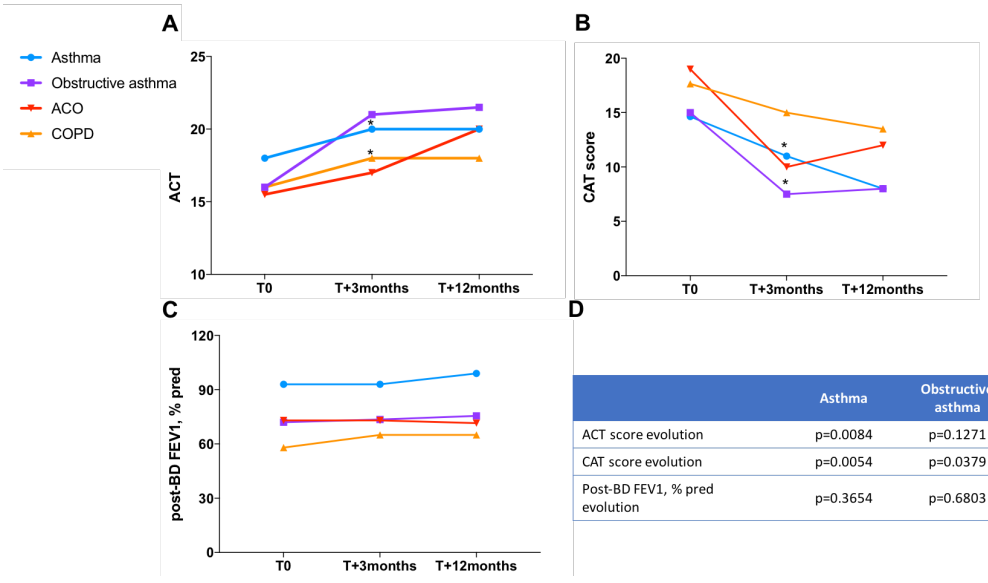


Results of agreement between physician on visit 2 and algorithm expressed on a Visual analogue scale (VAS). Every colored symbol represents a subject and colored horizontal lines represent median values. The black line represents a significant difference obtained by Kruskal-Wallis test with Dunn's post-hoc test, *p<0.05.

Evolution of disease

The within-group evolution of symptom scores between visit 1, visit 3 and visit 4 is presented in Figure 9. Asthma and COPD had a significant increase in ACT, whereas asthma and obstructive asthma demonstrated a significant decrease in CAT over time. The evolution of post-BD FEV1 over time was not significant in none of the groups. The between-group differences in change of ACT, CAT and post-BD FEV1 between visit 1, visit 3 and visit 4 were not different, as were the numbers of reported exacerbations (Table 4).

Figure 9: Evolution of symptom scores and FEV1 within algorithm-defined groups



D

	Asthma	Obstructive asthma	ACO	COPD
ACT score evolution	p=0.0084	p=0.1271	p=0.0861	p=0.0387
CAT score evolution	p=0.0054	p=0.0379	p=0.0611	p=0.0587
Post-BD FEV1, % pred evolution	p=0.3654	p=0.6803	p=0.7827	p=0.5547

Evolution of ACT (A), CAT (B) and post-BD FEV1 (C) from inclusion to visit 3 (T+3months) and visit 4 (T+12months). Panel D shows p-values of the Kruskal-Wallis tests on these data. *represents significance in this group.

Table 4: Change in symptom scores and FEV1, and number of exacerbations compared between algorithm-defined groups

	ASTHMA	Obstructive asthma	ACO	COPD	Control	p-value
ACT V3-V1	3 (-1 - 5)	4 (-0.5 - 8.5)	2 (-1 - 8)	1 (-1 - 3)	2 (2 - 2)	0.6330
CAT V3-V1	-2.5 (-8 - 2)	-5.5 (-9.8 - -2.3)	-6 (-11 - 1)	-1.5 (-6 - 2.3)	-3.5 (-5 - -2)	0.1763
ACT V4-V1	3.5 (0 - 5.9)	3 (-1 - 5.3)	6 (0 - 7)	1 (0 - 4)	6 (5 - 9)	0.1861
CAT V4-V1	-6 (-19 - -1.3)	-2 (-9 - -1)	-8 (-9 - 1)	-1 (-9 - 1)	-11 (-14 - -6)	0.1099
ΔPost-BD FEV1 V3-V1, %pred	-1 (-4.5 - 6)	2.5 (-8 - 13.8)	4 (0 - 5)	2 (-2 - 7.3)	5 (-1 - 17)	0.6188
ΔPost-BD FEV1 V4-V1, %pred	-1 (-4 - 7)	1.5 (-5.3 - 24.3)	-3 (-5.3 - 3.8)	3.5 (-4.3 - 8.3)	0 (-15.8 - 3.8)	0.6026
≥1 exa/y, %	38.9	35.7	63.6	47,8	NA	0.4362
≥2 exa/y, %	25.0	35.7	45.5	26.1	NA	0.5273

Results are expressed as median change (Q1-Q3) or %. Analysis were performed with Kruskal-Wallis test or chi^2. V1: visit 1, V3: visit 3, V4: visit 4, exa: exacerbation, y: year.

Analysis of the algorithm by supervised machine learning

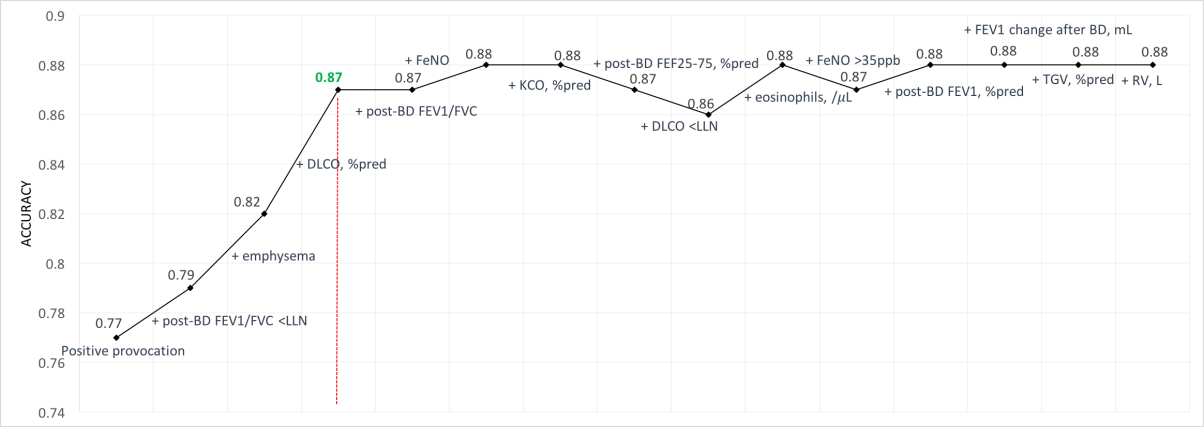
The contribution of the different variables to the diagnosis was investigated by machine learning strategies. Out of all available variables, 15 were calculated that contributed most (Table 5) and used to build a new, as accurate as possible, model. Interestingly, a maximum accuracy of 87% was reached with 4 parameters only, namely the presence of positive provocation, post-BD FEV1/FVC <LLN, emphysema on chest CT and % pred of DLCO. Adding other variables to the model did not significantly increase the accuracy (Figure 10). Figure 11 visualizes how the groups are clustered based on this model. Unfortunately, this model showed a 0% sensitivity for ACO in contrast to sensitivities of 100% for asthma, 83% for obstructive asthma, 96% for COPD and 93% for control.

Table 5: Variables that contributed most to diagnosis

1. Positive provocation
2. Post-BD FEV1/FVC, <LLN
3. Emphysema on chest CT
4. DLCO, %pred
5. Post-BD FEV1/FVC
6. FeNO, ppb
7. KCO, %pred
8. Post-BD FEF25-75, % pred
9. DLCO, <LLN
10. Eosinophils, /µL
11. FeNO, >35ppb
12. Post-BD FEV1, % pred
13. FEV1 change after BD, mL
14. TGV, % pred
15. RV, L

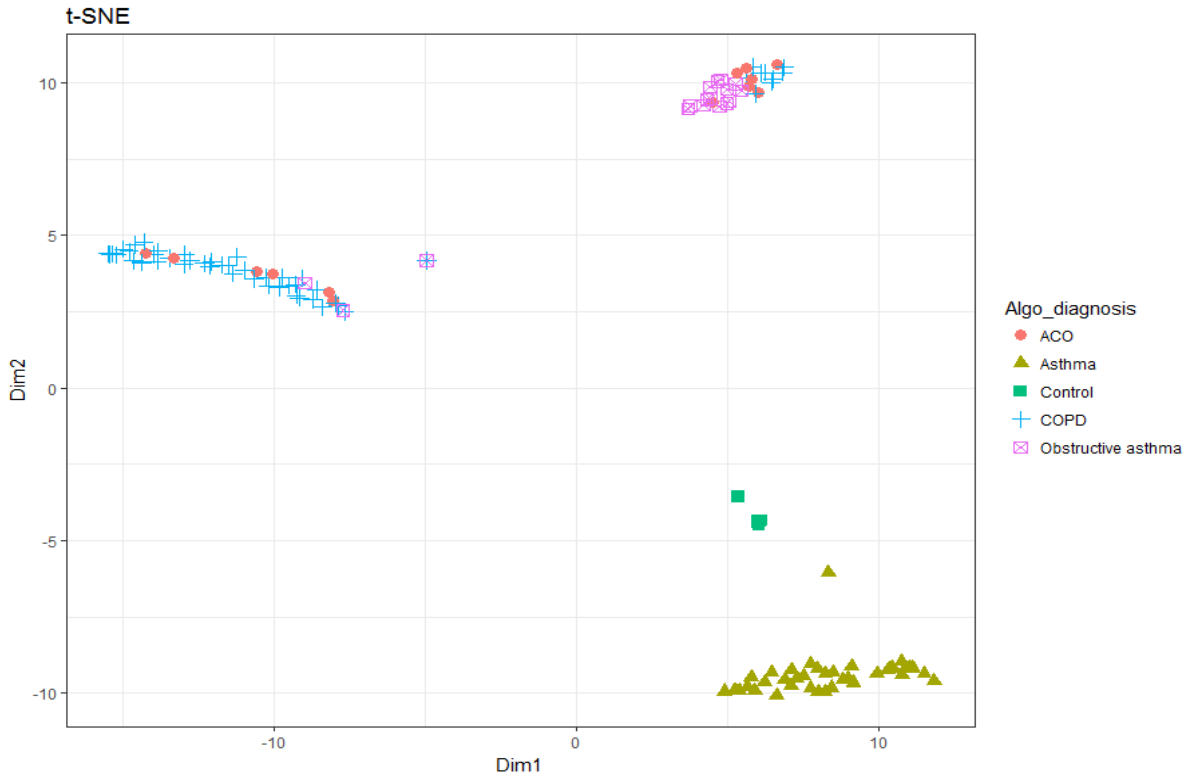
BD: bronchodilator, FEV1: Forced expiratory volume in 1 second, FVC: Forced vital capacity, LLN: Lower limit of normal, DLCO: Diffusion capacity of the lung for carbon monoxide, FeNO: fractional exhaled nitric oxide, KCO: carbon monoxide transfer coefficient, FEF25-75% forced expiratory flow between 25-75%, TGV: thoracic gas volume, RV: Residual volume

Figure 10: Step-wise build-up model and accuracy of the model per added variable



Random forest plots and 5-fold cross-validation were used to obtain these models. At every point a variable was added. An accuracy of 87% was reached with 4 variables, after which a plateau was reached and adding other variables made no significant difference.

Figure 11: Clusters based on computer-driven 4-variable model



t-SNE plot showing how diagnoses cluster, based on the 4-variable model developed by supervised machine learning.

DISCUSSION

The diagnostic algorithm that was used, was able to assign patients with obstructive lung disease to 4 groups. Symptoms, questionnaires and basic spirometry did insufficiently discriminate between groups, which emphasizes the need for additional tests. Markers for emphysema, namely DLCO and chest CT, and presence of Th2 inflammation contributed most to the differentiation of obstructive diseases. Machine learning was able to create a model with a minimum of variables, although not accurate for ACO.

Mainly observational, retrospective studies have been published on this topic, next to an abundance of reviews. To our knowledge, our study is the first of its kind, trying to prospectively differentiate patients with obstructive lung disease, including longitudinal follow-up. In our study population, demographics, such as age and sex, did not differ between groups. Within the obstructive groups (obstructive asthma, ACO and COPD) respiratory symptoms, degree of airflow limitation and RV/TLC as a measure of hyperinflation did not differ. The finding of respiratory symptoms not contributing to differential diagnosis, is in line with previous results (29), although higher scores on the Saint George's Respiratory Questionnaire have been demonstrated in ACO (30,31).

In contrast, the difference between the obstructive groups was mainly found in the presence of emphysema, either on chest CT or reflected by DLCO, and Th2 signature, which is plausible and deliberately instructed by the algorithm. Both FeNO and blood eosinophils were clearly highest in obstructive asthma and ACO. Sputum eosinophils, to which we were blinded throughout the study, confirmed this finding. Taken together, this difference in Th2 signature between groups was an important feature in differential diagnosis and therefore determinative for therapy. Without additional investigations, many patients may have been treated unnecessarily with ICS, if compared to the algorithm. Nevertheless, more than 30% of ACO subjects had neutrophilic or paucigranulocytic sputum results, whereas almost 38% of COPD subjects had sputum eosinophilia, demonstrating that also algorithm-based therapy might not be perfect and possibly over- and undertreated some patients with ICS. Prior reports showed a good correlation between blood and sputum eosinophilia (32), and these biomarkers have been suggested good predictors for ICS response in COPD (5,6). Although ICS have been shown clearly less effective in non-eosinophilic asthma (33,34), the presence

of sputum or blood eosinophilia is not used to guide therapy in the current asthma guidelines and ICS remain the mainstay of asthma treatment, regardless of the underlying endotype (8). Which approach is appropriate for ACO is largely unknown and a challenging topic for future research.

Due to unfullfilment of criteria, the algorithm was unable to attribute a diagnosis in 8% of subjects with obstructive spirometry. The test criteria for obstructive asthma may be to strict and focus mainly on the eosinophilic asthma phenotype. On the other hand, the use of LLN as cut-off for obstructive airflow, in order to reduce under-diagnosis in younger patients and over-diagnosis in the elderly (36), may have complicated the algorithm diagnosis. During expert panel discussion, we came across patients with different features of COPD, mainly emphysema, although FEV1/FVC was above LLN. In case of DLCO, we noticed that use of LLN is extremely sensitive, resulting in a high number of patients with DLCO below LLN, even in non-obstructive groups. At the time our study started, the new reference values of the Global Lung Initiative were not applied yet (22), which may explain some of these discordances.

Another remarkable finding in the study is the phenotype of the asthma group. None of these patients had an obstructive spirometry pre-BD. They all had a positive provocation test, but only a small subset demonstrated reversibility and increased FeNO compared to obstructive asthma and ACO. Additionally, this group does not represent early-onset asthma as confirmed by the low percentage of childhood asthma. Instead, the majority of these patients had adult-onset asthma, including all inflammatory phenotypes as reflected by the differential cell counts on sputum (eosinophilic, neutrophilic, paucigranulocytic and a minority with mixed granulocytic cells).

Although many patients were already using inhalation therapy at inclusion, therapy was adjusted based on the algorithm diagnosis. The results of longitudinal follow-up showed a general improvement in symptom scores for all groups, whereas FEV1 remained stable. Because of drop-outs, sample sizes were lower in these sub-analyses, which probably underpowered the results. The need for therapy changes during the study has to be investigated. With regards to the agreement between algorithm and expert panel, 21% of

cases received a diagnosis that deviated from the one proposed by the algorithm with the highest disagreement for obstructive asthma. This finding highlights the relevance of repetitive testing and evaluation of therapy response in diagnosing obstructive airway disease. Unfortunately, clinical evolution over time is often not accounted for in diagnostic criteria and clinical studies.

Supervised machine learning techniques identified the most contributing variables. Surprisingly, an accurate model was developed without Th2 variables. Nevertheless, the accuracy was mainly present for asthma, COPD, control and to a lesser extent for obstructive asthma. The computer-driven model was unable to recognize ACO. This emphasizes the complexity of this clinical problem and the reason why this entity is so hard to define. Anyhow, these techniques provide opportunities for better discrimination of obstructive lung diseases based on minimal information, leading to more precise medicine. Further analyses and optimization of the model will be performed and adjusted to expert diagnoses.

Although the use of diagnostic labels is currently under debate, we do need a consistent approach and a phenotypic description of obstructive lung diseases in order to investigate and include subgroups in further studies, resulting in accurate precision medicine.

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Chapter 6

General discussion

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1. Summary of results and general remarks

In the first part of this thesis, the role of *A. fumigatus* in COPD patients with bronchiectasis was investigated. Sensitization to this fungus was found to be more prevalent in COPD compared to controls and significantly associated with the presence of bronchiectasis. This was especially the case for specific recombinant antigens. Next, a more abundant presence of *A. fumigatus* was prospectively shown in sputum samples of COPD patients with bronchiectasis by galactomannan detection, which turned out to be a much more sensitive method than the routinely used cultures. Moreover, the administration of systemic CS was an important risk factor for increased galactomannan values in patients with bronchiectasis.

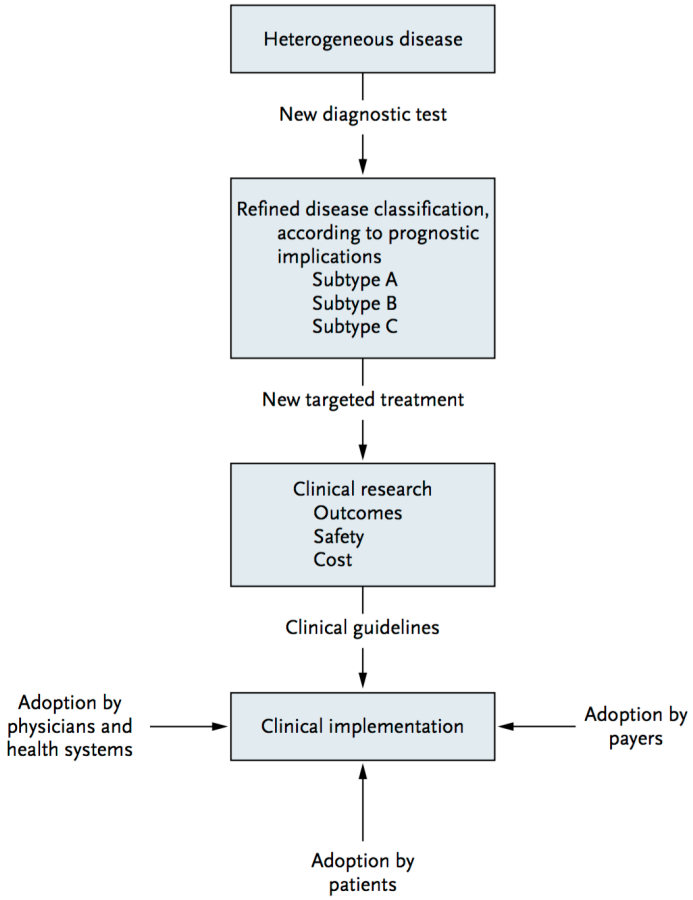
In the second part of the thesis, differences in the bronchial tree of COPD patients with and without bronchiectasis and controls were investigated by advanced imaging studies that covered the entire zone of conducting airways. COPD lungs with bronchiectasis demonstrated significantly more large and small airways compared to COPD lungs without bronchiectasis, although no difference in numbers of terminal bronchioles, which are the last segments of the conducting zone, were shown.

The last part of this thesis consists of a prospective clinical study aiming to differentiate distinct obstructive airway diseases, including asthma-COPD overlap. A pre-defined diagnostic algorithm was able to guide us in assigning patients to different groups, with presence of a Th2 signature and/or emphysema as the most important features for discrimination. Nevertheless, the algorithm has some limitation and could be improved with the help of supervised machine learning (artificial intelligence).

The main reason for investigating differences between patients with COPD is the pursuit of the most optimal therapy for an individual patient. Precision medicine has been proposed the driver for advances in heterogeneous diseases and defined as "treatments targeted to the needs of individual patients on the basis of genetic, biomarker, phenotypic or psychosocial characteristics that distinguish a given patient from other patients with similar clinical presentation"(1) (Figure 1). This translates into improving clinical outcomes and

minimizing unnecessary side effects (2). In fact, this modern concept of precision medicine could already have been derived from the age-old Hippocratic Oath (400 BC) we traditionally take, after graduating from our medicine studies.

Figure 1: Precision medicine



Schematic representation of the successive steps leading to precision medicine. Reproduced with permission from (1), Copyright Massachusetts Medical Society.

All research in this thesis was performed in order to achieve a better understanding of phenotypes, in a disease where therapeutic strategies currently have only a limited effect on evolution. Despite maximum available and evidence-based therapy, many patients do daily suffer from COPD symptoms and improvements in outcome have stalled. Of course, we realize that this work is only a small step in an immense and complex field and it only looked into the very first steps of the precision medicine process (Figure 1). Nevertheless, we hope the results may contribute to the unraveling of some underexplored potential phenotypes of COPD. Because of the high prevalence and a huge burden worldwide, we should definitely

continue investigating and trying to understand all aspects of COPD. Hopefully this will lead to new treatable traits and an improved quality of life in many patients.

In this general discussion, focus will be on future perspectives, unanswered questions and research opportunities within the different topics, since every chapter of this thesis already includes a specific discussion on the performed study.

2. COPD and *Aspergillus fumigatus*

Is A. fumigatus relevant, apart from bronchiectasis?

An association between *A. fumigatus* and bronchiectasis was demonstrated and discussed in chapter 3, but we assume that fungi are relevant in patients with COPD, also regardless of bronchiectasis. The role of fungi in COPD is underexplored and gives rise to many questions. Firstly, fungal presence in COPD airways must be largely underestimated, due to the poor sensitivity of fungal cultures. Other non-invasive techniques should be urgently optimized to act as a biomarker and correctly estimate the burden of fungi in chronic respiratory diseases. Next to chronic and invasive aspergillosis, which is beyond the scope of this thesis, *Aspergillus* is probably capable of causing non-invasive tracheobronchitis that may be responsible for uncontrolled respiratory symptoms. In one out of few studies about this topic, bronchiectasis and COPD were the most common underlying diseases (3). *Aspergillus* bronchitis has also been described in CF (4,5). Whether this entity of superficial fungal tracheobronchitis exists and contributes to COPD, is a question we should tackle with well-designed studies using bronchoscopy, histology and sensitive detection techniques. In this perspective, galactomannan detection on respiratory samples and/or *A. fumigatus* specific IgG are potential biomarkers that deserve further investigation.

Why are patients with COPD susceptible to A. fumigatus?

An intriguing and fundamental theme to explore in future research projects, is the susceptibility of COPD patients to *Aspergillus*. Next to the evident reasons, such as structural lung damage and reduced mucociliary clearance, other factors may contribute to susceptibility. Alveolar macrophages show a reduced ability to phagocyte bacteria in COPD

(6), and this is probably also the case for fungal spores. Wu et al showed a reduced clearance of *A. fumigatus* conidia in cigarette smoke exposed rats (7) and very recently, Wrench et al demonstrated a defective in vitro clearance of fungal spores by macrophages of smokers and COPD patients (8). Mechanism underlying these findings are unclear, but hopefully these results lead to more fundamental research to elucidate the host-fungus interactions in COPD. Finally, patients with specific gene polymorphisms may be more prone to *A. fumigatus* colonization or infection. A significant association between a polymorphism within pentraxin-3, a soluble pattern recognition receptor with an important role in innate defense against *Aspergillus*, and pulmonary aspergillosis has been shown. Furthermore, higher pentraxin-3 plasma levels were measured in these subjects (9). So far, this is the only report about underlying genetic susceptibility to *A. fumigatus* in COPD, but there is undoubtedly more to discover.

Are there therapeutic implications?

So far, studies about antifungal treatment in colonized and sensitized patients (with or without suspicion of tracheobronchitis) are very limited and mainly performed in asthma and CF. Some show promising results (5,10), others are rather disappointing due to limited effect or recurrence of fungi after treatment (3,11,12). Therapeutic implications deserve further study, taking also risks and side effects of antifungal therapy into account. We should even ask ourselves if there may be a role for preventive measures. A recent paper demonstrated an association between CS treatment and increased fungal burden in asthma patients with ABPA or *Aspergillus* sensitization (13). Future research should confirm the influence of CS on fungal burden in COPD, as suggested in chapter 3B, and guide us in administration and dosing of CS (both inhaled and systemic) in patients with known *Aspergillus* colonization and sensitization. Presence of *A. fumigatus* specific IgG, as demonstrated in chapter 3A, may highlight the impact of exposure. Maybe we should consider to reduce fungal exposure in patients with chronic respiratory disease by educating them about fungal sources (such as compost heap, hay, etc.).

Are there any other interacting factors in this field?

The clinical relevance of *A. fumigatus* in COPD is not elucidated, but the topic becomes even more complex if we speculate on interactions with other PPM in the airways. Although *A. fumigatus* and *P. aeruginosa* are often isolated at the same time in individuals with bronchiectasis, studies have suggested that *P. aeruginosa* is capable of inhibiting *A. fumigatus* growth (14,15). Nevertheless, one report demonstrated reduction of *Aspergillus* presence after intravenous antibiotics in CF (16). These contradictory findings are probably due to the capability of biofilm formation by both pathogens. *P. aeruginosa* is a clinically relevant and common colonizer in patients with bronchiectasis, so a better understanding of interactions and biofilm formation could help us optimize treatment and prevention strategies. Moreover, patients with COPD have an increased risk for nontuberculous mycobacterial (NTM) infection (17,18), with ICS as an important risk factor. Interestingly, NTM are a particular cause of bronchiectasis and a link between NTM and *Aspergillus* sensitization or ABPA has been shown (19,20). Moreover, the macrolide azithromycin could be an additional connection between *A. fumigatus* and NTM. In CF, the use of azithromycin has been reported a risk factor for sensitization to *A. fumigatus* (21,22) and for development of ABPA, whereas long-term macrolide treatment, as prescribed in frequent exacerbating COPD patients, may induce macrolide resistance in NTM. It would be of major interest to discover interactions between fungi and NTM and investigate the role of CS and azithromycin with regards to these pathogens.

3. COPD and bronchiectasis

Does A. fumigatus have a causal role?

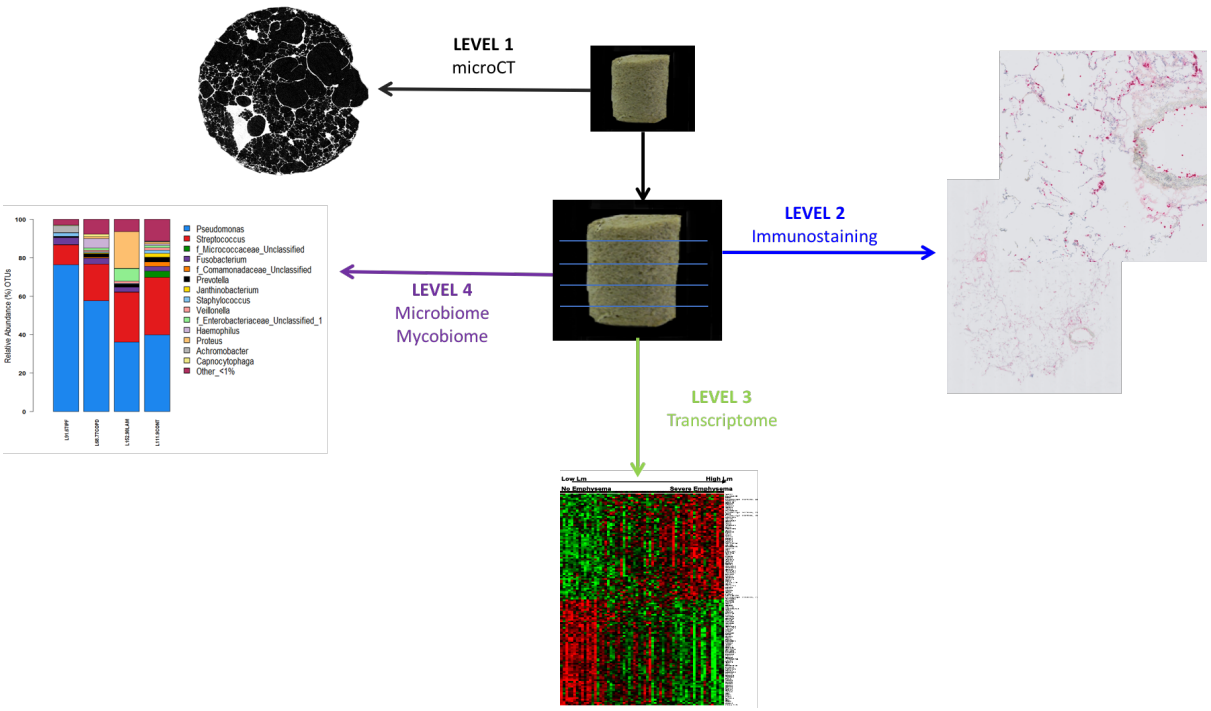
The studies performed in chapter 3A and 3B were merely explorative and not designed to demonstrate causality between *Aspergillus* and bronchiectasis in COPD. In chapter 4, a role of *Aspergillus fumigatus* in the COPD lungs with bronchiectasis was not investigated, as sputum samples of these patients were not available. Sample sizes in this study were also too small to confirm associations, furthermore galactomannan detection and *Aspergillus* PCR are not validated for tissue. Nevertheless, we attempted to detect *Aspergillus* on tissue cores of patients with confirmed colonization, but results turned out negative, illustrating that these

techniques are not optimized yet for application on tissue. Next to the methodology, it may be that the fungal load in the distal airways is below the detection limit of these techniques. As discussed below, next generation sequencing techniques (mycobiome) may solve this problem in the future. The absence of models for airway colonization with hyphae in immunocompetent mice is a general limitation in the study of host-fungus interactions and airway remodeling (23). To our knowledge, only one group established such a model (24). Hopefully the findings in chapter 3 will convince researchers to put effort into mechanistic research about the link between fungi and bronchiectasis, which is missing at this moment.
Obviously, also clinical, longitudinal studies may be able to show effects of *A. fumigatus* on airway remodeling, but these will need much more time as both microbial diagnostics and imaging have to be performed serially, in a standardized way over a long period of time and in a considerable number of patients with COPD. Nevertheless, existing datasets on COPD cohorts, that include imaging, may provide opportunities.

What are the other differences in COPD lungs with bronchiectasis?

The morphometry study in chapter 4 is only a first step in determining differences between COPD patients with versus without bronchiectasis. A systems biology approach, including imaging, immunohistochemistry, microbiome and RNA profiling on tissue cores of these explanted lungs is ongoing (Figure 2). Immunostaining for neutrophils (MPO), lymphocytes (CD4, CD8 and CD20), macrophages (CD68), eosinophils (ECP), mast cells (CD117) and collagen (type I and III) has been performed. This will allow us to detect bronchiectasis-related differences in some innate and adaptive immune cells. For instance, we assume a more abundant presence of neutrophils in COPD lungs with bronchiectasis. An interesting topic in this perspective is the presence of different lymphocytes and especially the formation of lymphoid follicles. Recognized in COPD as a cause for small airway obstruction (25) and suggested in a subgroup of patients with bronchiectasis (26), the investigation of these follicles and their composition in patients with both COPD and bronchiectasis would add some novel findings to the field.

Figure 2: Future systems biology approach



The use of a single tissue sample for distinct analyses will provide integration of data from different levels and contribute to understanding of underlying pathways.

The role of micro-organisms and their interactions in different COPD phenotypes deserves further attention. The lung microbiome could be important in the crosstalk between respiratory tract pathogens and host response, since bacteria interact with each other and respond to altered environmental conditions. Furthermore, a difference in microbiome composition between bacterial and eosinophilic exacerbations has been shown (27). This emphasizes the potential of the lung microbiome as a biomarker to practice precision medicine in patients with COPD. Fungal biome -or mycobiome- research is only in its infancy and is more advanced yet in gastroenterology compared to pneumology. Fungal dysbiosis in the gut has been shown to aggravate intestinal inflammation and even exacerbated the development of allergic airway disease (28). Similar phenomena are not unlikely in the respiratory system. Bacterial and fungal DNA extraction from COPD tissue samples is challenging, but the technique is currently being optimized and hopefully we will be able to investigate microbial and fungal differences within COPD subgroups.

Transcriptome analysis (collaboration with N. Kaminski, Yale school of Medicine, New Haven, USA), may reveal differences in molecular pathways such as fibrosis, vasculature

development, antimicrobial defense including specific anti-fungal response, adaptive immune responses, etc. We aim to find differences in pathways involved in tissue repair, that may explain differences in airway remodeling between patients with versus without bronchiectasis. Moreover, single-cell RNA-sequencing may be helpful in more detailed analysis of regulatory differences between COPD patients with different phenotypes in future projects.

Are there other questions to answer with morphometry?

The cross-sectional investigation of explanted lungs will further evolve, as more detailed analysis of the bronchial tree, such as measurement of segment lengths and bifurcation angles, is feasible and will reveal additional anatomical differences between distinct COPD phenotypes. Our lab just succeeded in microCT scanning of large specimens in frozen state, allowing to conserve the lungs for in-depth investigation (genetic, microbial etc.) after scanning, to integrate high resolution imaging with –omics data. Moreover, segmental quantification of emphysema and its relation with regional bronchiectasis could reveal hints on the association between emphysema and bronchiectasis in COPD. In alpha-1 antitrypsin deficiency, the relation between emphysema and bronchiectasis seems proportional (29) and in post-infective or idiopathic bronchiectasis, the number of bronchopulmonary segments with emphysema is associated with clinical severity (30). Such statements cannot be made about COPD, where the relation between emphysema and bronchiectasis is hardly investigated.

Longitudinal analysis of airway count and dimensions within individual patients would be of major value to demonstrate that the composition of the bronchial tree contributes to the expression of certain phenotypes such as bronchiectasis. As HRCT is highly accessible in daily practice and radiation exposure is getting less, we are optimistic that we will be able to study patients with COPD that underwent CT imaging on distinct moments over the course of their disease. This approach will teach us where and when airways exactly alter and contribute substantially to the understanding of the pathophysiology of COPD.

4. Asthma-COPD overlap

Why is this topic so controversial?

First of all, the interplay of asthma and COPD in one individual is not understood. In the first Global Initiative for Asthma/GOLD report on asthma-COPD overlap in 2014, the term Asthma-COPD Overlap Syndrome (ACOS) was used to describe these patients. The consideration of labelling patients with different phenotypes as having the same syndrome caused a lot of criticism, and that is why this term was replaced by ACO in more recent literature. In fact, ACO is an umbrella term for patients with distinct phenotypes of obstructive lung disease. It includes smoking asthma patients with irreversible obstructive spirometry and COPD patients with increased blood eosinophils or very positive bronchodilator test (31). This is also the reason that no consistent definition is agreed upon. It is hard to make robust statements about a heterogeneous group and this creates a lively debate among experts.

Do all ACO patients need ICS?

A common treatment strategy is the main rationale for assigning a diagnostic label to a group of patients. A lot of evidence exists on the administration of ICS in asthma, where benefits outweigh risks (32,33). In recent years, the use of ICS in COPD is debated, since advantages may not outweigh the risks of particularly pneumonia (34), but also osteoporosis, cataracts and oropharyngeal candidiasis. However, blood eosinophilia has been put forward as an important biomarker for both exacerbation risk (35,36) and corticosteroid responsiveness in COPD (35,36). The evidence on the relation between eosinophils and ICS response is mainly derived from retrospective analyses on large randomized controlled trials. The majority used blood eosinophils ≥2% (corresponding to ~150 cells/μL) as a good predictor for ICS response, defined as a significant reduction in number of exacerbations (36,37), but other cut-offs have been suggested (38,39). It is not clarified yet if the cut-off to predict exacerbations and the one to predict ICS response are the same, as is the use of % versus cells/μL. Many patients with COPD have eosinophils ≥2%; for instance only 13.6% of COPD subjects in the ECLIPSE study had <2% of blood eosinophils on áll visits (40). Eosinophil-associated airway inflammation is probably not limited to asthma and therefore this 2% cut-off is -in our opinion- not sufficient to consider these patients as having ACO.

Previous studies (41) as well as our study in chapter 5, showed that patients with ACO have an increased Th2 signature with higher eosinophils compared to COPD. This suggests that these patients would benefit from ICS treatment. However, only a few, small studies investigated the efficacy of ICS in ACO (42–45), showing beneficial effects. One of them reported in favor of the potential for blood and sputum eosinophilia to predict ICS responsiveness (42), another showed no link between ICS response and blood eosinophils (45). The rationale for ICS use in ACO is mainly deduced from knowledge in asthma and COPD with high eosinophils, rather than based on evidence and large, randomized studies. Furthermore, not all patients with ACO will present with a high Th2 profile. If smoking asthma patients with obstructive airflow are considered ACO, not all will exhibit eosinophilic inflammation, since asthma, especially in smokers, may also present with neutrophilic or paucigranulocytic inflammation (32). This has been shown in previous studies (41,46) and is also clear from our study in chapter 5; although a high Th2 signature was shown in the ACO group, 30% of these patients had non-eosinophilic sputum results. Although neutrophilic asthma phenotypes have been shown to respond less to ICS (47,48), current asthma guidelines do not use inflammatory endotypes to guide ICS treatment and consider ICS the mainstay of treatment in all asthma patients. A last remark in this context is the probability that patients with ACO have some corticosteroid resistance, such as patients with severe asthma, smoking asthmatics and COPD. According to professor Barnes, this corticosteroid resistance is an important, potential therapeutic target in itself (49).

In conclusion, the answer to this question is far from assured yet, although the evidence in asthma and COPD suggests the need for ICS in ACO, with eosinophils as a potential biomarker. Further studies are definitely needed to confirm the assumed need and safety of ICS in ACO.

Will we find a specific biomarker for ACO?

It seems an impossible task to discover an ACO-specific biomarker. As described before, ACO represents different phenotypes and is not solely based on Th2 inflammation, therefore we will not discuss blood or sputum eosinophils and FeNO in this paragraph. Some studies investigated differences between biomarker profiles in asthma, COPD and ACO, but in the majority a significant difference was only found between two of the three groups. So far, the

sputum level of neutrophil gelatinase-associated lipocalcin, a neutrophil derived molecule also expressed by respiratory epithelial cells, has been shown the only marker able to distinguish patients with asthma, COPD and ACO, with the highest levels in the latter group (50,51). This has to be confirmed in more and larger studies.

Markers of systemic inflammation (IL-6, IL-8 and tumor necrosis factor-α), Th2-related markers (periostin, IL-5 and IL-13) and Il-17 were determined in serum of patients with asthma, COPD and ACO. Surprisingly, none of these could individually differentiate one from the other two groups (46) nor predict a Th2-high inflammatory pattern. A panel or network of biomarkers for ACO may be more feasible. However, the same study showed very limited discrimination abilities by combinations of markers with discriminant analysis (46). So far, no ACO-specific biomarker(s) have been validated, and in our opinion it will be a very challenging quest. Nevertheless, we could not find any reports on exhaled breath condensate or organic volatile compound analysis, which may be helpful and provide opportunities for future research projects.

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SUMMARY - SAMENVATTING

SUMMARY

Chronic obstructive pulmonary disease (COPD) is a common disease, in the Western world mainly caused by exposure to cigarette smoke in a susceptible host. It is characterized by persistent respiratory symptoms and diagnosis is based on irreversible airflow limitation. In the last decade, it has become widely accepted that the degree of airflow limitation is insufficient to estimate severity and heterogeneity of this disease. Next to the intensity of symptoms, exacerbations and comorbidities, certain clinical phenotypes are related to different outcomes.

In this doctoral project, two underexplored potential phenotypes of COPD were investigated. Firstly, the presence of bronchiectasis. We highlighted the role of sensitization to *Aspergillus fumigatus (A. fumigatus)* as a possible risk factor for the development of bronchiectasis in COPD. Next, we demonstrated a higher load of *A. fumigatus* in sputum samples of patients with COPD ánd bronchiectasis with a novel, sensitive technique. Moreover, the administration of systemic corticosteroids in these patients was identified as an important risk factor for *A. fumigatus* in these patients. Apart from *A. fumigatus,* we revealed with novel imaging studies that the number of both large and small airways was clearly less reduced in patients with COPD and bronchiectasis versus patients without bronchiectasis in comparison with controls, except for the terminal bronchioles that were equally reduced in both COPD groups. This finding emphasizes that the composition of the bronchial tree may give rise to certain phenotypes.

Asthma-COPD overlap (ACO), a controversial, poorly defined entity, is another phenotype we took a closer look at. We propectively tested the accuracy of a diagnostic algorithm in obstructive lung disease and showed similarities and differences between groups. Symptoms, questionnaires and basic spirometry did insufficiently distinguish obstructive groups, which emphasizes the need for additional tests. Presence of emphysema and Th2 signature seemed to contribute most. We aim to further optimize a diagnostic model with the help of artificial intelligence.

The findings in this thesis deserve further investigations and confirmation. We hope they may be a first step in better understanding of these phenotypes, which may lead to unravelling of endotypes and identification of therapeutic targets, resulting in more precise medicine for patients with COPD.

SAMENVATTING

Chronisch obstructief longlijden (COPD) is een veelvoorkomende ziekte die in de westerse wereld voornamelijk voorkomt bij vatbare rokers. De ziekte wordt gekenmerkt door aanhoudende respiratoire symptomen en gediagnosticeerd door aanwezigheid van onomkeerbare luchtwegvernauwing. Het voorbije decennium is duidelijk geworden dat de graad van luchtwegobstructie onvoldoende is om de ernst en de heterogeniteit van de ziekte te beschrijven. Naast ernst van symptomen, exacerbaties en comorbiditeiten, zijn ook bepaalde klinische fenotypes gerelateerd aan een andere uitkomst.

In dit doctoraatsproject werden twee onderbelichte, potentiële fenotypes van COPD onderzocht. Een eerste is de aanwezigheid van bronchiëctasieën. We benadrukten de rol van sensitizatie tegen *Aspergillus fumigatus* (*A. fumigatus*) als een mogelijke risicofactor voor het ontwikkelen van bronchiëctasieën. Daarnaast toonden we met een nieuwe, gevoelige techniek een grotere aanwezigheid aan van *A. fumigatus* in sputumstalen van patiënten met COPD én bronchiëctasieën. Bovendien werd het gebruik van systemische corticosteroïden geïndentificeerd als een belangrijke risicofactor voor *A. fumigatus* in deze patiënten. Onafhankelijk van *A. fumigatus* ontdekten we met vernieuwende beeldvorming dat het aantal grote en kleine luchtwegen duidelijk minder afgenomen was in COPD patiënten mét versus zonder bronchiëctasieën in vergelijking met controles, uitgezonderd voor de terminale bronchiolen die in gelijke mate afgenomen waren in beide COPD groepen. Deze bevinding benadrukt dat de samenstelling van de bronchiaalboom aanleiding kan geven tot bepaalde fenotypes.

Astma-COPD overlap (ACO), een controversiële en slecht gedefinieerde entiteit, is een ander fenotype dat we onder de loep namen. We testten prospectief de accuraatheid van een diagnostisch algoritme in obstructieve longziekten. Symptomen, vragenlijsten en spirometrie konden de obstructieve groepen onvoldoende onderscheiden, wat de noodzaak van additionele testen aantoont. De aanwezigheid van emfyseem en Th2 inflammatie leken het meest bij te dragen. We beogen om een diagnostisch model te optimaliseren met de hulp van artificiële intelligentie.

De bevindingen in deze thesis verdienen verder onderzoek en bevestiging. We hopen dat ze een eerste stap kunnen zijn naar een beter begrip van deze fenotypes, wat kan leiden tot het

ontrafelen van endotypes en het ontdekken van therapeutische aanknopingspunten, resulterend in een preciezere behandeling van patienten met COPD.

SCIENTIFIC ACKNOWLEDGEMENTS,

PERSONAL CONTRIBUTION AND CONFLICT OF INTEREST

SCIENTIFIC ACKNOWLEDGEMENTS

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Many thanks to all the collaborators outside Leuven for the realized and future projects; Prof. Tim Nawrot (Hasselt University), Iván Josipovic and Prof. Matthieu Boone (Ghent University), Prof. James C Hogg (Vancouver, Canada) and Prof. Naftali Kaminski (Yale, USA).

PERSONAL CONTRIBUTION TO THE THESIS MANUSCRIPT

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Chapter 3A

Stephanie Everaerts collected all clinical data, prepared blood samples for analyses, scored all CT images, performed statistical analyses, interpreted results and drafted the manuscript.

Chapter 3B

Stephanie Everaerts included all patients, acquired clinical data, performed all sputum inductions, prepared, processed and interpreted results of sputum samples. Stephanie Everaerts performed statistical analyses and drafted the manuscript.

Chapter 4

Stephanie Everaerts helped in collecting and processing lungs, counted and measured airway bifurcations on CT images, performed airway segmentations and modeling on microCT images. She analyzed data, interpreted results and drafted the manuscript.

Chapter 5

Stephanie Everaerts helped in acquisition of data and organization of the dataset, analyzed the data, interpreted results and drafted the preliminary manuscript.

CONFLICT OF INTEREST

None of the people who worked on this thesis had any relevant conflict of interest with respect to the content. Stephanie Everaerts was supported by the Fonds Wetenschappelijk Onderzoek (FWO 12G8715N), Flanders. The laboratory of pneumology (Wim Janssens) is supported by the KU Leuven Research Fund (C24/15/030) and the Astra Zeneca Chair in respiratory pathophysiology KU Leuven.

ADDENDUM

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

The aging lung: tissue telomere shortening in health and disease

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Background

Telomere shortening has been associated with several lung diseases. However, telomere length is generally measured in peripheral blood leucocytes rather than in lung tissue, where disease occurs. Consequently, telomere dynamics have not been established for the normal human lung nor for diseased lung tissue. We hypothesized an age- and disease-dependent shortening of lung tissue telomeres.

Methods

At time of (re-)transplantation or autopsy, 70 explant lungs were collected: from unused donors (normal, n = 13) and patients with cystic fibrosis (CF, n = 12), chronic obstructive pulmonary disease (COPD, n = 11), chronic hypersensitivity pneumonitis (cHP, n = 9), bronchiolitis obliterans syndrome (BOS) after prior transplantation (n = 11) and restrictive allograft syndrome (RAS) after prior transplantation (n = 14). Lungs were inflated, frozen and then scanned using CT. Four tissue cores from distinct lung regions were sampled for analysis. Disease severity was evaluated using CT and micro CT imaging. DNA was extracted from the samples and average relative telomere length (RTL) was determined using real-time qPCR.

Results

The normal lungs showed a decrease in RTL with age ($p < 0.0001$). Of the diseased lungs, only BOS and RAS showed significant RTL decrease with increasing lung age ($p = 0.0220$ and $p = 0.0272$ respectively). Furthermore, we found that RTL showed considerable variability between samples within both normal and diseased lungs. cHP, BOS and RAS lungs had significant shorter RTL in comparison with normal lungs, after adjustment for lung age, sex and BMI ($p < 0.0001$, $p = 0.0051$ and $p = 0.0301$ respectively). When investigating the relation between RTL and regional disease severity in CF, cHP and RAS, no association was found.

Conclusion

These results show a progressive decline in telomere length with age in normal, BOS and RAS lungs. cHP, BOS and RAS lungs demonstrated shorter RTL compared to normal lungs. Lung tissue RTL does not associate with regional disease severity within the lung. Therefore, tissue RTL does not seem to fully reflect peripheral blood telomere length.

Respir Res. 2018 May;19 (1):95.

Trials in pulmonary rehabilitation

The author performed 45 muscle biopsies in COPD patients that were included in the subsequent trials:

Enhancement of training response following pulmonary rehabilitation with downhill walking: a randomized controlled trial. Supervisors: Prof. T. Troosters and Prof. W. Janssens. Manuscript in preparation.

Enhancing physical activity to achieve sustainable benefits in extrapulmonary consequences of COPD: a randomized parallel group trial. Supervisors: Prof. T. Troosters and Prof. W. Janssens. Work in progress.

The Belgian trial with Azithromycin for acute COPD Exacerbations requiring hospitalization (BACE)

The author protocoled 490 ECGs of patients with COPD, performed in the context of this trial.

Supervisor: Prof. W. Janssens

COPD airway microbiota: application of next-generation sequencing to characterize the viral components during acute exacerbations.

The author collected 14 sputum samples of COPD patients during hospitalization and performed 9 sputum inductions in healthy controls. Composition of airway virome will be detected on these samples.

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Everaerts S, Lammertyn EJ, Martens DS, De Sadeleer LJ, Maes K, Dupont LJ, Wuyts WA, Vos R, Gayan-Ramirez G, Kaminski N, Hogg JC, Janssens W, Verleden GM, Nawrot TS, Verleden SE, McDonough JE, Vanaudenaerde BM. The aging lung: tissue telomere shortening in health and disease. Respir Res. 2018 May 11;19(1):95.

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Abstracts presented at international conferences (only first author)

Everaerts S, Verleden SE, McDonough JE, Serré J, Mathyssen C, Boone M, Josipovic I, Dupont LJ, Vanuadenaerde BM, Janssens W. Airway morphometry in COPD-bronchiectasis overlap. Poster discussion European Respiratory Society Annual Congres 2018, Paris.

Everaerts S, Lagrou K, Dupont L, Vanaudenaerde B, Janssens W. Sputum galactomannan in COPD patients with and without bronchiectasis. Thematic poster European Respiratory Society Annual Congress 2017, Milan.

Everaerts S, Lagrou K, Dubbeldam A, Lorent N, Vermeersch K, Van Hoeyveld E, Bossuyt X, Dupont LJ, Vanaudenaerde BM, Janssens W. Sensitization to Aspergillus fumigatus as a risk factor for bronchiectasis in COPD. Oral presentation European Respiratory Society Annual Congress 2017, Milan.

Everaerts S, Vermeersch K, De Bent K, Denaux K, Verschueren S, Lorent N, Vanbleyenbergh P, Dupont L, Janssens W. Prospective diagnostic algorithm to differentiate asthma, ACOS and COPD. Poster discussion American Thoracic Society International Conference 2017, Washington DC. *ATS scholarship*

Everaerts S, Vermeersch K, Van Hoeyveld E, Vanaudenaerde B, Bossuyt X, Lagrou K, Dupont L, Janssens W. Sensitization to Aspergillus fumigatus in COPD patients with and without bronchiectasis. Thematic poster 1st World Bronchiectasis Conference 2016, Hannover.

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Oral presentations at national conferences

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